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CANCER RESEARCH

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Further Evidence for Maternal Effect and Modifiers of Tumorous Head Genes in Natural Populations of *Drosophila melanogaster**

ELDON J. GARDNER AND MORRIS D. GARDNER

(*Utah State Agricultural College, Logan, Utah*)

An inherited abnormality (Figs. 1–9), consisting of irregular growths of various sizes in the head region of the tu^h strain of Drosophila, is under investigation. The strain was obtained from Dr. Wilson Stone and appeared in 1945 in a culture of flies obtained at Acahuizotla, Mexico, in 1941 by a collecting party from the University of Texas. Two interacting genes, *tu-1* in the first chromosome and *tu-3* in the third chromosome, are responsible for the trait (8). Since *tu-1* or its alleles has been found in several natural populations, it is possible that this gene was already present in the Mexico collection and that only *tu-3* arose by mutation. The descriptive term "tumorous head,"[1] symbolized tu^h, has been used to identify the trait and the flies which carry the genes. The description of the abnormality (4, 8, 13), genetic mechanism (8), influence of temperature on expression (9), and relative viability (5) of tumorous head flies have been presented. A maternal effect controlled by *tu-1* was interpreted from the results of reciprocal outcrosses (8).

Both wild and mutant stocks from the laboratory were tested for alleles and modifiers of susceptibility genes. Three of 21 wild stocks (6) carried alleles of *tu-1*. Modifiers increasing the percentage of abnormal flies from 1 to 10 per cent in the F_2 were found in one mutant and three wild stocks. Both modifiers and alleles seemed to be homozygous. Modifiers were found in certain individuals in all eight natural populations tested (7). The purpose of the present paper is to report the frequency of tumor genes in certain natural populations widely distributed in the United States and four foreign countries and to present further evidence for a maternal effect.

MATERIALS AND METHODS

Flies from 28 natural populations, 24 in the United States and four in foreign countries, were collected[2] with nets and traps from garbage cans, fields, and fruit stands. The locations in the United States from which the collections were obtained are indicated in Chart 1. The samples of flies were introduced into vials 9½ cm. high × 2½ cm. in diameter containing about 8 cc. of the standard cornmeal, agar, and molasses media and transported to the laboratory. About 25 males taken at random from each of the original collections or first generation cultures were mated individually in culture vials with tu^h females. About 25

* This investigation was supported in part by the Damon Runyon Memorial Fund and in part by a grant-in-aid from the American Cancer Society upon recommendation by the Committee on Growth of the National Research Council.

[1] The term "tumor" is used in a broad sense to identify abnormal growths. No relationship with vertebrate neoplasms is implied. Histological and cytological data concerning "tumorous head" will be reported in subsequent papers.

Received for publication March 27, 1953.

[2] The collections from Berkeley, Knight's Landing, and Linden, California, were made by D. D. Jensen, Associate Professor of Entomology, University of California. Those from Farmington, Utah, Grand Junction and Palisade, Colorado, were made by Howard Dorst, Entomologist, United States Department of Agriculture. The Los Angeles collection was obtained from Wayne Stokes; the Willard collection from Mr. and Mrs. Jack Barker; and the Ogden sample from Dean A. Gardner. The collections from foreign countries: Argentina, China, Crimea, and Formosa were received from Ernest Jund, Department of Genetics, University of California. All others were collected by the authors.

virgin females were taken as they appeared from the first generation culture, prepared from each collection, and mated individually with tu^h males. In diagrams of crosses the female parent is always written first. The F_1 flies were classified for the presence of abnormal growths. Several pairs of F_1 flies from each culture were mated to produce the F_2 progeny which were also examined and classified. The temperature was controlled at 22° ±2° C. The percentages of abnormal flies were summarized with accuracy to the nearest whole number.

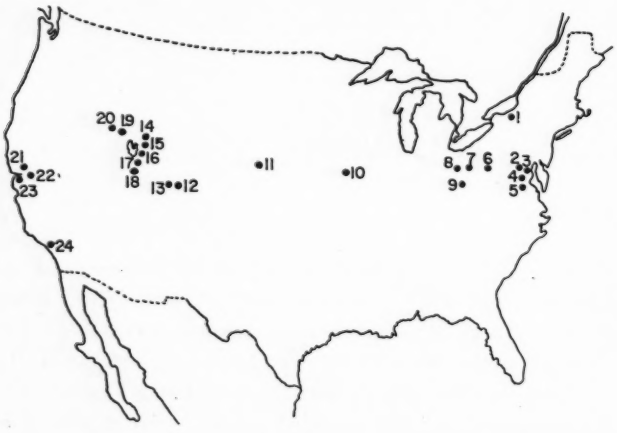

CHART 1.—Locations of natural populations sampled in the United States. 1. Auburn, 2. Frederick, 3. Baltimore, 4. Alexandria, 5. Mount Vernon, 6. West Newton, 7. Cambridge, 8. Columbus, 9. Greenfield, 10. Corydon, 11. North Platte, 12. Palisade, 13. Grand Junction, 14. Logan, 15. Willard, 16. Ogden, 17. Farmington, 18. Salt Lake City, 19. Burley, 20. Twin Falls, 21. Knight's Landing, 22. Linden, 23. Berkeley, 24. Los Angeles.

The F_1 data obtained from the control crosses and 1,331 reciprocal pair matings between tu^h flies and those collected from natural populations are summarized in Table 1. An average of 30 per cent of F_1 progeny from the pair matings between tu^h females and Lausanne males were abnormal. From the second control cross between tu^h females and Canton males 49 per cent were abnormal. All F_1 progeny from 30 reciprocal matings with Lausanne and 28 with Canton were normal. The general pattern for the experimental crosses was comparable to that of the controls. Deviations were attributed to the presence of modifiers in the different population. From a combined total of 55,111 F_1 flies from the cross between tu^h females and wild males, 11,377 or 20.6 per cent were abnormal. From the 75,626 classified from the reciprocal cross, 808 or 1.07 per cent were abnormal. The combined results of the crosses from the 24 samples in the United States, illustrating the maternal effect, are shown graphically in Chart 2.

The F_2 results from the control and experimental crosses are summarized in Tables 1 and 2. All 28 of the wild populations sampled showed evidence of segregating modifiers. From the com-

TABLE 1

SUMMARIZED RESULTS OF RECIPROCAL PAIR MATINGS BETWEEN tu^h FLIES AND THOSE COLLECTED FROM NATURAL POPULATIONS

	F_1		F_2	
Cross	Total flies	Av. per cent abn.	Total flies	Av. per cent abn.
tu^h×Lausanne (control)	2185	30	3029	0
Lausanne×tu^h (control)	1995	0	1050	0
tu^h×Canton (control)	2497	49	2277	0
Canton×tu^h (control)	4460	0	2767	0
tu^h×Auburn	2163	21	2598	2
Auburn×tu^h	3159	0	3182	2
tu^h×Frederick	1861	20	2404	8
Frederick×tu^h	2430	5	2805	4
tu^h×Baltimore	1716	14	2967	3
Baltimore×tu^h	2563	2	2808	4
tu^h×Alexandria	1855	9	2546	0
Alexandria×tu^h	2372	0	2736	1
tu^h×Mt. Vernon	1894	24	2129	5
Mt. Vernon×tu^h	2908	0	3354	5
tu^h×West Newton	1406	25	2608	1
West Newton×tu^h	2328	1	2658	2
tu^h×Cambridge	2692	20	2893	2
Cambridge×tu^h	2910	1	3701	3
tu^h×Columbus	2718	17	3330	0
Columbus×tu^h	2523	0	3307	0
tu^h×Greenfield	2221	15	2600	2
Greenfield×tu^h	2752	0	3507	1
tu^h×Corydon	1557	23	2246	0
Corydon×tu^h	2557	0	2648	0
tu^h×North Platte	1698	24	2335	1
North Platte×tu^h	2770	0	2851	2
tu^h×Palisade	2026	20	3055	1
Palisade×tu^h	2884	0	3468	2
tu^h×Grand Junction	1390	20	2111	12
Grand Junction×tu^h	4504	4	6102	8
tu^h×Logan	2867	12	3758	2
Logan×tu^h	2766	0	4214	1
tu^h×Willard	2585	15	2947	5
Willard×tu^h	3343	2	3032	5
tu^h×Ogden	2178	18	2826	4
Ogden×tu^h	2892	0	3188	3
tu^h×Farmington	1678	25	2918	3
Farmington×tu^h	2624	2	2801	6
tu^h×Salt Lake City	2255	31	3143	0
Salt Lake City×tu^h	2982	1	4180	2
tu^h×Burley	1816	20	2447	0
Burley×tu^h	2649	1	3029	2
tu^h×Twin Falls	1874	31	2869	1
Twin Falls×tu^h	2839	6	3218	4
tu^h×Knight's Landing	2281	22	1076	1
Knight's Landing×tu^h	2730	0	1080	0
tu^h×Linden	526	13	722	1
Linden×tu^h	888	0	793	1
tu^h×Berkeley	2119	19	3531	0
Berkeley×tu^h	2625	0	3300	2
tu^h×Los Angeles	1435	19	1155	3
Los Angeles×tu^h	2633	0	1981	1
tu^h×Argentina	1312	22	1717	2
Argentina×tu^h	3018	0	3222	2
tu^h×China	2085	33	2670	1
China×tu^h	2426	0	2816	0
tu^h×Crimea	2547	26	2694	1
Crimea×tu^h	1805	1	2599	3
tu^h×Formosa	2351	27	1375	0
Formosa×tu^h	2746	0	2915	1

bined total of 69,670 F_2 flies from the original cross between *tu*^{h} females and wild males, 1,472 or 2.1 per cent were abnormal. From the reciprocal cross, 2,176 or 2.5 per cent of the 85,495 F_2 flies classified were abnormal. The comparable results of the reciprocal crosses indicate that the maternal effect is not carried over to the F_2. The gene *tu-1* must be completely recessive, and no substance influencing the maternal effect is retained by the F_1 mothers.

The variations in percentage of abnormal flies attributed to modifiers were discontinuous, suggesting a distinct effect of single genes segregating in the natural populations. The elusive nature of the modifiers made classification and identification of their position on the chromosomes impossible. However, genes from wild populations were combined in the same flies with the *tu*^{h} genes through selection from the progeny of experimental crosses. Lines were thus synthesized in which 90–100 per cent of the flies were abnormal, compared to 76 per cent from the inbred *tu*^{h} stock. Samples from nine wild populations: Frederick, Baltimore, Mount Vernon, Grand Junction, Willard, Ogden, Farmington, Burley, and Twin Falls carried genes which behaved as if they might be alleles of *tu-1*. Since modifiers in other loci could produce the same results, it was impossible to determine, on the basis of these experiments, which were actually alleles.

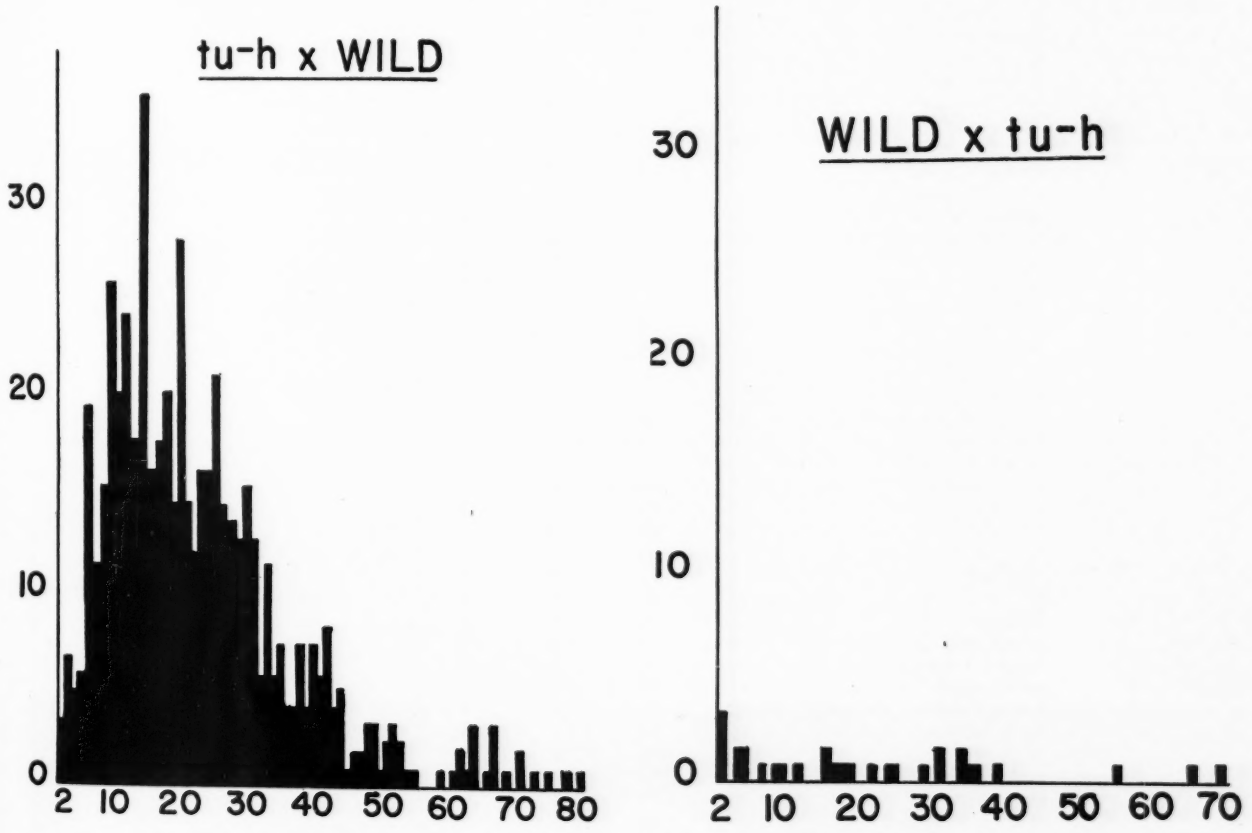

CHART 2.—Combined results from 1131 reciprocal pair matings between *tu*^{h} flies and samples from 24 natural populations in the United States illustrating maternal effect. The abscissa indicates the percentage of abnormal flies in each culture in which abnormal flies appeared; the ordinate, the number of cultures.

DISCUSSION

Other phenotypes bearing some resemblance to tumorous head have been described. Zimm (21) investigated a mutant much like tumorous head which was identified as an allele of Lobe (Lobe^D). Flies carrying Lobe alleles have been compared and crossed with tumorous head, but no genetic relationships have been detected. Sobels (16) investigated the genotype "asymmetric" which produced some manifestations similar to those of tumorous head, but abnormalities of the thorax and abdomen were also included. This genotype has not been compared experimentally to tumorous head. Another phenotype somewhat like tumorous head was received through Dr. Bentley Glass from Hans-Joachim Becker. It reacted as if it might have a modifier of tumorous head but showed no evidence of allelism or further inter-

action. The common mutants: eyeless, bar, deformed, kidney, and aristapedia resemble some expressions of tumorous head but have no genetic relationship. The mutant erupt described by Glass (10) is the only one investigated which shows a genetic relationship with tumorous head. Phenotypically erupt resembles one expression of tumorous head, but the abnormal growth is restricted to the eyes. The two stocks (carrying tumorous head and erupt) have something in common, but the nature of the genetic relationship has not been established.

TABLE 2

ANALYSIS OF F_2 RESULTS OF RECIPROCAL PAIR MATINGS BETWEEN *tu*^{h} FLIES AND THOSE COLLECTED FROM NATURAL POPULATIONS

		Per cent abnormal flies in cultures					
Cross	Matings	0–1	2–4	5–14	15–24	25–34	35 or above
tu^h×Lausanne	29	29					
Lausanne×*tu*^{h}	26	26					
tu^h×Canton	13	13					
Canton×*tu*^{h}	19	19					
tu^h×Auburn	21	17	2	1	1		
Auburn×*tu*^{h}	24	12	7	5			
tu^h×Frederick	20	8		6	5	1	
Frederick×*tu*^{h}	22	14	2	5		1	
tu^h×Baltimore	22	7	11	4			
Baltimore×*tu*^{h}	21	10	7	3	1		
tu^h×Alexandria	22	19	2	1			
Alexandria×*tu*^{h}	22	17	4	1			
tu^h×Mt. Vernon	19	10	1	6	2		
Mt. Vernon×*tu*^{h}	26	6	6	13	1		
tu^h×West Newton	22	19	1	1	1		
West Newton×*tu*^{h}	21	16	2	3			
tu^h×Cambridge	24	16	5	3			
Cambridge×*tu*^{h}	27	18	5	3	1		
tu^h×Columbus	25	24	1				
Columbus×*tu*^{h}	26	24	1	1			
tu^h×Greenfield	25	22	1	1		1	
Greenfield×*tu*^{h}	26	22	2	2			
tu^h×Corydon	20	19		1			
Corydon×*tu*^{h}	22	22					
tu^h×North Platte	16	15			1		
North Platte×*tu*^{h}	25	17	3	5			
tu^h×Palisade	24	20	2	2			
Palisade×*tu*^{h}	25	16	4	5			
tu^h×Grand Junction	9	4	1	1		1	2
Grand Junction×*tu*^{h}	29	13	4	7		4	1
tu^h×Logan	24	22		1		1	
Logan×*tu*^{h}	25	21	3	1			
tu^h×Willard	25	14	3	5	2	1	
Willard×*tu*^{h}	29	20	1	6	1		1
tu^h×Ogden	24	19	1		3	1	
Ogden×*tu*^{h}	25	17	1	6	1		
tu×Farmington	25	18	1	5	1		
Farmington×*tu*^{h}	24	10	3	8	1	2	
tu^h×Salt Lake City	24	23	1				
Salt Lake City×*tu*^{h}	25	19	2	3	1		
tu^h×Burley	19	18	1				
Burley×*tu*^{h}	24	20	1	2	1		
tu^h×Twin Falls	22	20		1	1		
Twin Falls×*tu*^{h}	26	20		2	3	1	
tu^h×Knight's Landing	23	17	3	2	1		
Knight's Landing×*tu*^{h}	24	24					
tu^h×Linden	20	14	3	3			
Linden×*tu*^{h}	30	27	1	2			
tu^h×Berkeley	25	24	1				
Berkeley×*tu*^{h}	25	21		4			
tu^h×Los Angeles	17	7	6	4			
Los Angeles×*tu*^{h}	24	19	5				
tu^h×Argentina	19	12	5	2			
Argentina×*tu*^{h}	28	13	12	3			
tu^h×China	26	23	3				
China×*tu*^{h}	25	24	1				
tu^h×Crimea	25	17	7	1			
Crimea×*tu*^{h}	26	11	10	5			
tu^h×Formosa	20	19	1				
Formosa×*tu*^{h}	23	21	2				

FIGS. 1–9.—Expressions of tumorous head

Hereditary internal melanotic growths have been investigated extensively in Drosophila, since they were first described by Bridges (1). Many investigators, including Stark (17, 18), Wilson (19), Russell (14, 15), Hartung (11), Burdette (2, 3), and Herskowitz and Burdette (12), have reported on such growth abnormalities. Six Drosophila stocks carrying genes for internal melanotic tumors were compared to and crossed with tumorous head (6). No similarity or genetic relationship was detected.

One problem which requires further discussion is the reason for the apparently homozygous condition of alleles of *tu-1* and modifiers of the *tu*^{h} genes in laboratory stocks, compared to the varying frequency of these genes in wild populations. The three wild laboratory stocks (Oregon R, Stephenville, and Crimea) which were found to carry *tu-1* alleles were apparently homozygous for these genes. Likewise, the four laboratory stocks found to carry modifiers of *tu-3* were uniform and homozygous. Other laboratory stocks were uniformly free from alleles and modifiers. All wild and mutant laboratory stocks originated ultimately from collections in natural populations. All the samples taken in the present study carried alleles or modifiers or both, but none was uniform in this respect. In natural populations the genes seem to be segregating freely, presumably at equilibrium. Different frequencies were indicated for the genes in different populations. The tumorous head phenotype has never been observed in a wild population. If it should occur it would not compete favorably in natural selection. The modifying genes alone have no visible expression and presumably no selective value. The method by which laboratory stocks are maintained may account for the tendency for genes with no selective value to be eliminated or become homozygous in the population. Small numbers of flies are transferred to new culture bottles between generations. Wright (20) has shown that genes may become established or eliminated by chance in small breeding populations. This so-called "genetic drift" may be the mechanism by which laboratory cultures become homozygous or free from genes segregating freely in natural populations.

SUMMARY

1. Twenty-eight populations from widely scattered geographical locations carried modifiers of genes for tumorous head in different frequencies, whereas in laboratory stocks previously tested such genes were either completely absent or homozygous.

2. Consistently different results in F_1 reciprocal crosses indicated a maternal effect. No difference was detected in the F_2 results of the reciprocal crosses.

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Free-Amino Acid Content and Transaminase Activity of Lymphatic Tissues and Lymphosarcomas*

SAUL KIT AND JORGE AWAPARA
WITH THE TECHNICAL ASSISTANCE OF SUE CURNUTTE

(The University of Texas, M. D. Anderson Hospital for Cancer Research, Houston, Texas)

The observed levels of free amino acids within a tissue reflect the capacity of the tissue to remove these substances from the blood, produce them from other carbon sources, and utilize them in various processes. Each tissue has a characteristic *pattern* (2, 15). Since the dynamic equilibria which determine the endogenous amino acid concentrations may be disturbed in disease, observation of the pattern of the pathological tissue may provide insight into the nature of the pathological condition itself.

It seemed particularly appropriate to investigate neoplasms and their normal homologs in this connection. Qualitative patterns of a number of neoplasms and normal tissues have already been published by Roberts and Frankel (15). In the present study, quantitative determinations were carried out on four lymphosarcomas, a transplantable methylcholanthrene-induced rat tumor, and normal lymphatic tissues of three animal species.

Since the transaminases seem to regulate the formation and utilization of amino acids from carbohydrate precursors, a cursory survey was also made of these enzymes.

METHODS

Healthy adult rabbits, C3H and Ak mice, and Sprague-Dawley and Wistar rats were used in these experiments. The rat and mouse thymi and spleens were from nontumor-bearing animals. It was necessary to pool two or three mouse spleens and five or six mouse thymi in order to provide enough tissue for an experiment. One animal provided enough tissue for each experiment in the case of the other tissues.

Two tumors were transplanted subcutaneously into each animal, and the tissues were used 12–14 days later in the case of the Gardner lymphosarcoma and 10–12 days later in the case of the other tumors.[1] At this time, each of the mouse tumors weighed about 300–400 mg., and there was no gross evidence of necrosis. Non-necrotic samples of the rat tumors (600–800 mg.) were taken. A description of the lymphosarcomas, including photographs of microscopic sections has been published by Sugiura and Stock (16, 17). It is to be noted that all but the Murphy-Sturm lymphosarcoma are composed almost exclusively of lymphocytes. The latter tissue contains an admixture of lymphocytes and muscle cells.

Fat and mucoid materials were trimmed away, and the tissues were quickly weighed and then homogenized in 20 cc. of 80 per cent ethanol. Centrifuge tubes containing the alcoholic solution were placed in a boiling water bath to complete the coagulation of protein. After centrifugation, the protein precipitate was discarded, and the alcoholic solution was extracted with chloroform (2). The water phase was separated from the chloroform and evaporated to dryness on a steam bath. The residue was taken up in a volume of water equivalent to the weight of the original tissue, and the resulting solution was filtered. Aliquots equivalent to from 30 to 80 mg. (usually 50 mg.) wet weight of tissue were used for chromatography.

Quantitative amino acid determinations were performed by the method of Awapara (2), except for the following modifications as suggested by Fowden (7); (*a*) after development with water-saturated phenol, the papers were dried at about 35° C. in a stream of air, and residual phenol was removed with ether; (*b*) the ninhydrin spots were cut from the paper, placed in test tubes, and after the addition of 0.1 cc. of 0.1 M NaOH, these test tubes were stored in an evacuated vacuum desiccator for 8 hours. Additional citrate was included in the Moore and Stein ninhydrin reagent (13) equivalent to the NaOH which had been added. By this procedure, reagent blanks against propanol-water were usually about 0.090 optical density units, and paper blanks about 0.140. Standards at three levels for glutamic, aspartic, glycine, and alanine were run on paper simultaneously with all tissue extract determinations. The optical density of 0.25 μM of glutamic acid was about 0.400.

Transaminases were measured by the method of Awapara and Seale (3), except that incubations were carried out for 30 minutes at 38° C. under N_2 in the Dubnoff apparatus. Experiments were terminated by the addition of 5 cc. of ethanol, and the contents of the beakers were transferred to 12-ml. centrifuge tubes. Beakers were rinsed with 2 cc. of ethanol, and the combined ethanolic solutions were boiled for 2 minutes to coagulate protein and prepared for chromatography as previously described. Quantitative determinations after chromatography with phenol-water were completed with the previously mentioned modification; an alkali treatment of the paper was used to minimize paper blanks. The determinations were performed in duplicate.

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[1] The authors are indebted to Dr. K. Sugiura of the Sloan-Kettering Institute for the gift of Ak mice bearing the Patterson and Mecca lymphosarcomas and the Wistar rats bearing the Murphy-Sturm lymphosarcoma. The methylcholanthrene-induced rat tumor was induced at this institution and was diagnosed as a carcinosarcoma.

RESULTS

Distribution of amino acids.—The distribution of free amino acids in various lymphatic tissues and in some lymphosarcomas is shown in Charts 1 and 2. Values are expressed as μM amino acid/gm wet weight of tissue. It should be noted that this form of expression does not correct for the relative cellularity per unit weight of each tissue. The reproducibility from experiment to experiment was relatively good, except in the case of those amino acids present at relatively low concentrations. The amino acid *pattern* of a given tissue, however, was found to be extremely constant.

As may be observed from the charts, the amino acid patterns of the normal lymphatic tissues resemble one another closely. The lymphosarcomas show characteristic patterns that are different in several respects from the normal lymphatic tissues and also from the carcinosarcoma. Chromatograms of the free amino acids from the lymphosarcomas are similar to the chromatogram of the transplantable mouse lymphosarcoma reported by

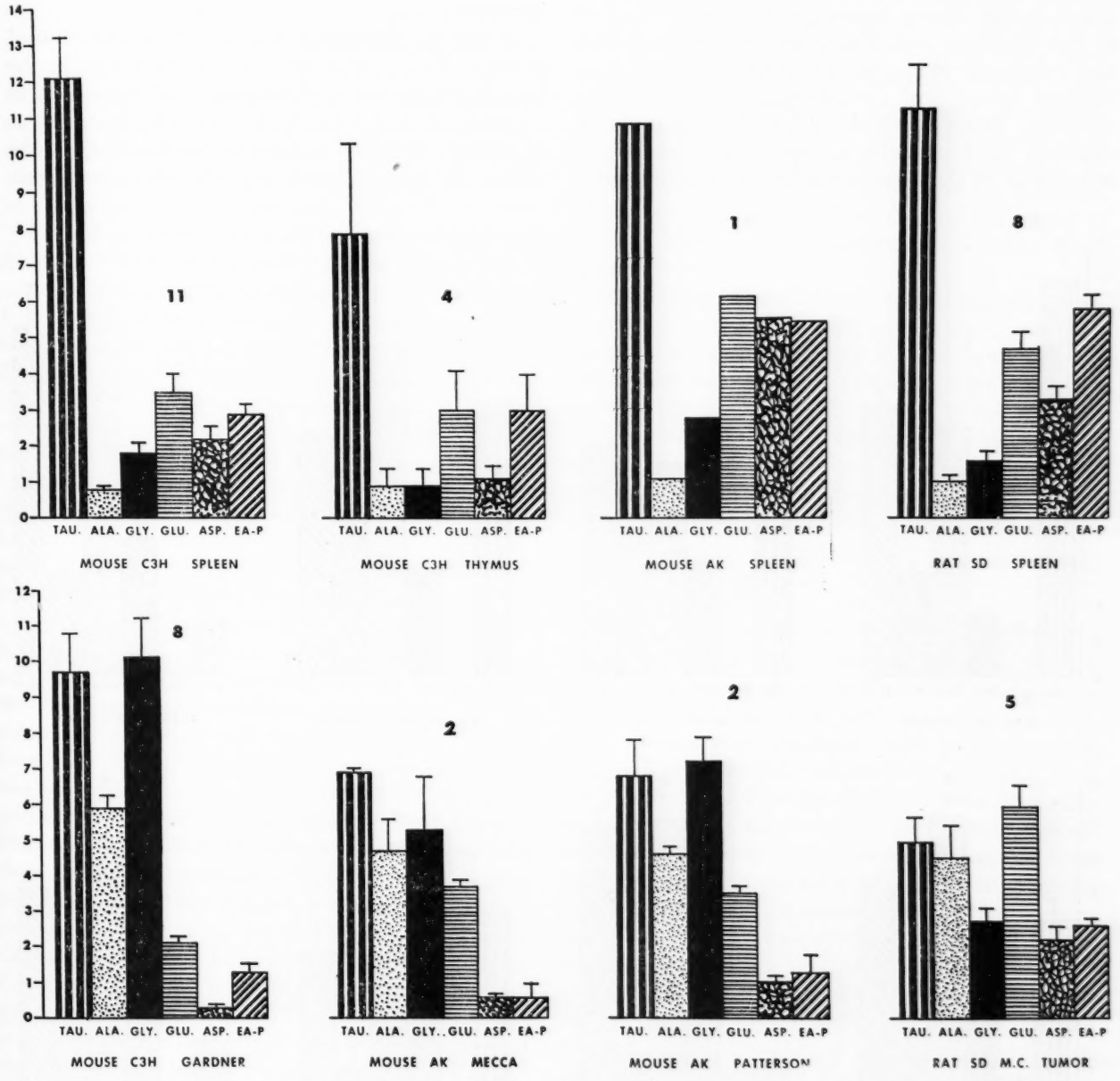

CHART 1.—Amino acid concentrations of lymphatic tissues and tumors.

Ordinate: μM per gram wet weight of tissue. Height of each bar represents the mean concentration. The number of determinations for each tissue is shown above each barograph. The standard error of the mean is shown above each bar.

Abbreviations are: Tau = Taurine; Ala = Alanine; Gly = Glycine; Glu = Glutamic acid; Asp = Aspartic acid; EA-P = Ethanolamine phosphoric ester; SD = Sprague-Dawley.

Roberts and Frankel (15), and the chromatograms from the normal lymphatic tissues resemble that published by Roberts and Frankel for a mouse lymph node.

All five tumors were found to contain high concentrations of alanine. This amino acid exists in normal lymphatic tissues in amounts corresponding to one-fourth of the concentration of glutamic acid, but in the lymphosarcomas the concentration of alanine attains values as high as 3 times the concentration of glutamic acid. The concentration of ethanolamine phosphoric ester is drastically reduced in the Mecca tumor, somewhat diminished in the Gardner and Patterson lymphosarcomas, and relatively lower in the methylcholanthrene-induced tumor and the Murphy-Sturm lymphosarcoma. Proline, which is found only in trace amounts in lymphatic tissues, was clearly detectable in all the tumors studied, when chromatograms were made with the equivalent of 50 mg. wet weight of tissue. Glutamine is usually present in concentrations similar to the concentration of alanine in most tissues but is reduced in the lymphosarcomas. The glycine content of the Mecca, Patterson, and Murphy-Sturm tumors is very high compared to the content of glutamic acid; in the Gardner lymphosarcoma the glycine concentration is about 5 times greater than the concentration of glutamic acid. The concentration of aspartic acid was found to be 4–5 times less than the concentration of glutamic acid in the lymphosarcomas.

A species difference was noted in the case of taurine. This metabolite is relatively low in the lymphatic tissues of the rabbit, but very high in rat and mouse lymphatic tissues. The low content of taurine may be ascribed to the inability of the rabbit to convert cysteine into this compound.

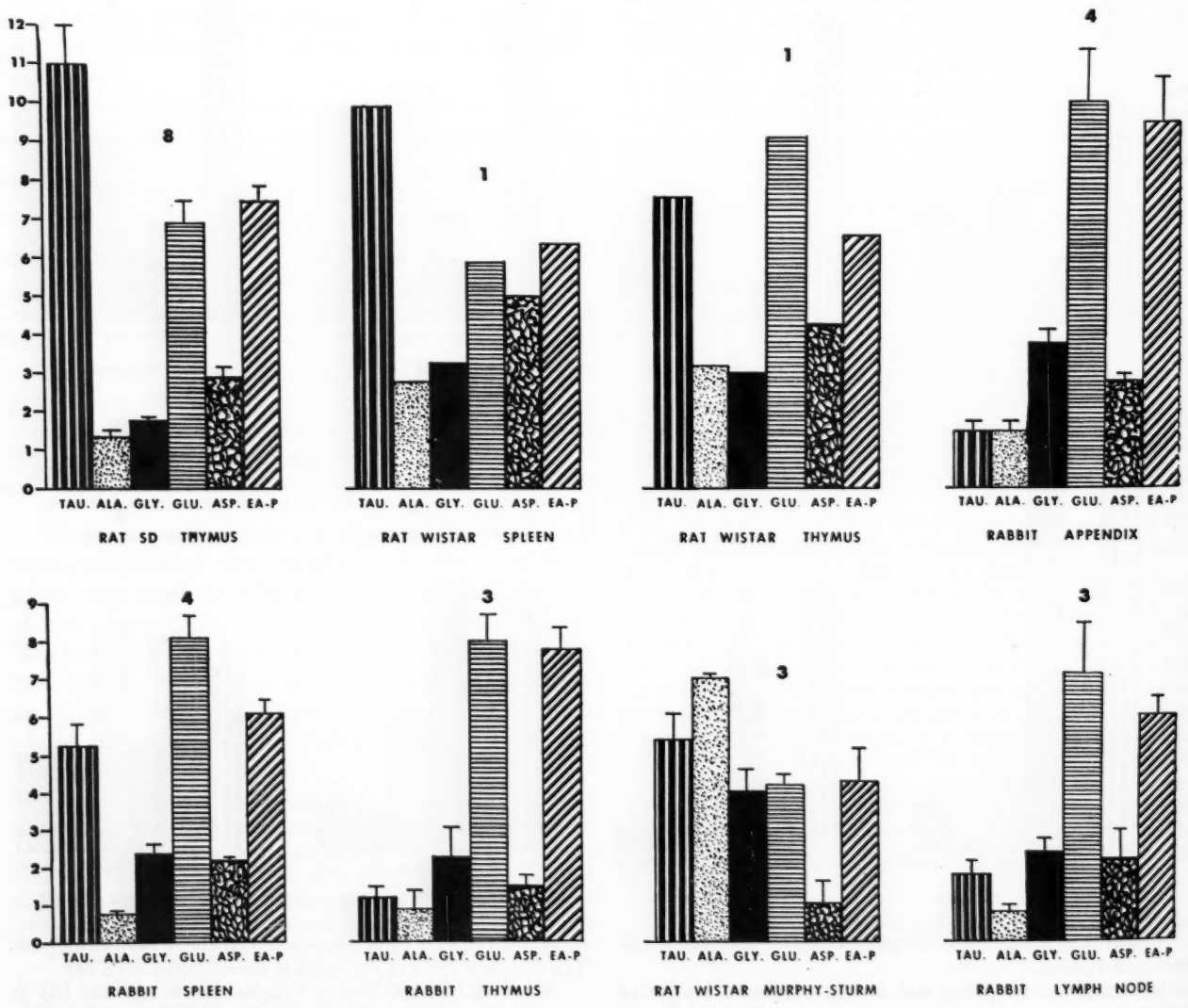

CHART 2.—Amino acid concentrations of lymphatic tissues and tumors. See Chart 1 for abbreviations.

Data obtained in this laboratory indicate that after cysteine is injected into the rabbit it is converted slowly and only in very small amounts into taurine in the liver, whereas in rat liver this conversion occurs rapidly and in substantial amounts (1, 4).

Amino acid pattern of solid and ascites tumor.—The Gardner lymphosarcoma will grow as an ascites tumor in C3H mice. It was of interest to compare the amino acid pattern of the ascites tumor with that of the subcutaneously grown Gardner tumor, which was used in the experiments reported above. Ascites tumors were therefore induced in two mice by intraperitoneal inoculation of tumor cells, and the animals were sacrificed 10 days later. The tumor cells were sedimented by centrifugation at 0° C. and the ascites serum discarded. The cells were extracted with 80 per cent ethanol, and the extract was prepared for chromatography as previously described.

The amino acid pattern of the ascites tumor was found to resemble the solid tumor. Thus, if one assumes that each cc. of sedimented cells weighed 1 gm., the concentrations in μM/gm wet weight for the ascites tumor were: taurine, 6.1; alanine, 5.6; glycine, 5.4; glutamic acid, 2.6; aspartic acid, 0.2; and ethanolamine phosphoric acid, 1.7. The corresponding values for the solid tumor were 9.7, 5.9, 10.1, 2.1, 0.3, and 1.3.

Thus, the concentrations of alanine and glycine were relatively high and aspartate and ethanolamine phosphoric ester relatively low. In addition, proline was readily detectable on the chromatograms, while glutamine was not present.

Transaminase activity.—All the normal lymphatic tissues and tumors possess active transaminases. In Table 1 are given values for some of the transaminase activities measured. The Q_T values (μl of amino acid formed/mg dry weight/hour) for the glutamic-oxalacetic transaminase was calculated to range from 42 for the appendix (rabbit) to 99 for mouse spleen. Redfield and Barron (14) reported Q_T values of 24 for appendix slices, while Cohen found a Q_T of 16 for rat spleen (6). The less active alanine-α-ketoglutaric transaminase was found in all the tissues. Very little alanine was formed, however, in the Gardner lymphosarcoma from pyruvate and glutamate or pyruvate and glutamine. The alanine-oxalacetic transaminase activity may be the summation of the alanine-α-ketoglutaric and the glutamic-oxalacetic transaminase activities. Active transamination between leucine, phenylalanine, and isoleucine with α-ketoglutaric acid was observed in the appendix (rabbit), spleen (rat), and Gardner lymphosarcoma. The values, not shown on the table, ranged from 11 to 89 μM of amino acid formed in 30 min. Further work on these transaminases is in progress.

DISCUSSION

The high concentrations of alanine in the tumors may be partially related to an increased tendency of certain tumors to concentrate amino acids (5). The same might apply to glycine or other amino acids. However, it seems likely that the high alanine concentrations are also attributable to an increased ability of these tissues to form alanine. The following observations support this hypothesis: (*a*) After tumor cell suspensions were incubated with carbon-14-labeled lactate (10), considerable radioactivity was found in alanine; (*b*) The incorporation of isotopic alanine into tumor cell protein *in vitro* (9) is markedly inhibited when pyruvate is added to the medium. This may be interpreted as due to a dilution of isotopic alanine with nonisotopic alanine from the pyruvate.

In addition, it is well known that the glycolysis of tumors is very high. Thus, pyruvate, the precursor of alanine, is rapidly formed in tumors. It was interesting to note, however, that, although appreciable amounts of alanine transaminated with α-ketoglutarate in the tumor homogenates, only a weak glutamine-pyruvate or glutamate-pyruvate transaminase was discernible. It is not clear whether other untested amino acids are the principal amino donors to pyruvate or whether other mechanisms than transamination are involved in alanine formation.

The low concentration of aspartic acid in the lymphosarcomas is of interest, since it was shown by Kit and Greenberg (10) that little radioactivity was to be found in aspartic acid after lymphosarcoma cells were incubated with labeled lactate, although considerable radioactivity was present in the aspartic acid fraction of spleen cells. It is difficult to reconcile the fact that glutamic-oxalacetic transaminase is very active in the tumor cells with the fact that aspartic acid exists in very low concentrations in the same cells. Further inquiry into this problem has revealed that considerable aspartic acid exists in bound form in these tumors. This could be shown by chromatography of the extracts before and after acid hydrolysis. Indeed, a fractionation of a strip chromatogram into six areas revealed that four of the six areas (none of which corresponded to the R_F of free aspartic acid) contained substances which, on hydrolysis, released aspartic acid. The nature of these substances is not known. It is possible, therefore, that the low levels of aspartate imply

rapid utilization of this amino acid. Aspartic acid or a related compound is a precursor of tissue pyrimidine (11).

The high concentration of glycine and relatively low content of ethanolamine phosphoric ester in the lymphosarcoma may also be related phenomena. Ethanolamine may be formed from glycine by way of serine (8, 12). The multiple functions of glycine in metabolic reactions are well known and necessitate no further comment.

Cohen (6) has reported that transaminase activity decreases during the induction of rat hepatomas by 4-dimethylaminoazobenzene. Transamination was generally low in a number of mouse tumors, and it was suggested that rapid growth or increased protein synthesis is associated with low transaminase activity. As shown in Table 1, this suggestion does not apply to lymphosarcomas when compared to normal lymphatic tissues.

TABLE 1

TRANSAMINASE ACTIVITY OF LYMPHATIC TISSUES AND TUMORS

Figures are means of duplicate determinations except where noted. The number of determinations made is shown in parentheses. 0.5 ml. of a 1:10 homogenate, 25 μM of keto acid, 25 μM of amino acid, and 0.01 M phosphate buffer, pH 7.4, to make a total volume of 3 cc. Incubation time: 30 minutes. Temperature: 38° C. Gas: N_2.

Tissue	Alanine–AKG*	Aspartic–AKG*	Alanine–OAA*	Glutamic–OAA*
Thymus (Wistar Rat)	15 (1)	68 (1)		208 (1)
Thymus (S.D. Rat)	21 (4)	121 (4)		
Spleen (Wistar Rat)	27 (1)	107 (1)	14 (1)	298 (1)
Spleen (S.D. Rat)	14 (4)	135 (4)		
Spleen (Ak Mouse)†	27 (1)	94 (1)	2 (1)	441 (1)
Spleen (C3H Mouse)	13 (2)	158 (2)	21 (2)	268 (2)
Appendix (Rabbit)	41 (1)	70 (1)	5 (1)	188 (1)
Murphy-Sturm Lymphosarcoma	13 (1)	195 (1)	2 (1)	317 (1)
Mecca Lymphosarcoma	58 (1)	198 (1)	12 (1)	255 (1)
Gardner Lymphosarcoma	38 (2)	136 (2)	29 (2)	286 (2)

Values are given in μM glutamic and/or aspartic formed per gram wet weight of tissue.

* AKG and OAA are α-ketoglutaric acid and oxalacetic acid, respectively.

† 1:20 homogenate.

SUMMARY

Quantitative measurements have been made of the amino acid content of four lymphosarcomas, a carcinosarcoma, and normal lymphatic tissues of rodents. The pattern was drastically altered in the neoplasms. The lymphosarcomas exhibit markedly elevated contents of alanine and glycine. Proline was also elevated in these tissues compared to the normal tissues. Diminutions were observed of aspartic, ethanolamine phosphoric ester, and glutamine. Measurements were also made of transaminase activity. The results were discussed in terms of the metabolic reactions which lead to the formation and utilization of these amino acids.

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Combination Chemotherapy of Cancer: Potentiation of Carcinostatic Action of 8-Azaguanine by a Riboflavin Analog*

L. S. Dietrich and Daniel M. Shapiro†

(Departments of Biochemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York, N.Y.)

Combination chemotherapy with 8-azaguanine and a noncarcinostatic riboflavin analog, 6-chloro-9-(1′-D-sorbityl)-isoalloxazine (flavotin), has been previously shown to produce greater carcinostasis than does 8-azaguanine alone (13). Similar augmentation of the carcinostatic action of 8-azaguanine was demonstrated with two other noncarcinostatic compounds, crude folic acid (14) and 6-formylpteridine (15). In the latter case, the pteridine derivative was shown to inhibit tumor guanase *in vitro*. On the basis of these findings, it was postulated that the potentiation of the carcinostatic action of 8-azaguanine observed *in vivo* was due to inhibition of 8-azaguanine deamination, since the deaminated product (8-azaxanthine) is without carcinostatic activity (5, 6). However, the results obtained with pteridine and with flavotin differ in two respects. First, the *in vitro* addition of flavotin had no effect on guanase activity. Secondly, the enhancement of 8-azaguanine carcinostasis produced by flavotin *in vivo* was reversed by riboflavin-5-phosphate (13). These observations indicated that flavotin might be acting as an antagonist of a flavo-enzyme dealing with purine metabolism, the only known possibility being xanthine oxidase.

The present study demonstrates the inhibitory effect of flavotin upon the xanthine oxidase activity of the 755 mammary carcinoma, and implicates this inhibition in a possible explanation for the enhanced carcinostasis produced by the combination of drugs.

MATERIALS AND METHODS

In vivo studies.—The 755 tumor, a mammary adenocarcinoma, was subcutaneously transplanted into the axillary region of female C57BL mice by the usual trocar technic. The mice, weighing 18–25 gm. and from 2 to 3 months of age, received an ad libitum diet of Rockland pellets and water. The preparation of the 8-azaguanine and flavotin for administration, their consistent carcinostatic effect upon the 755 tumor, the details of measuring tumor growth, and the method of statistical analysis have been previously described (13).

In vitro studies.—C57BL female mice were killed by cervical rupture. All animals received their last injection of flavotin 1 hour prior to being sacrificed. Guanase activity was measured as previously described (9, 15) by the micro-determination of the ammonia released by enzymatic action. Xanthine oxidase activity was followed manometrically using the procedure of Axelrod and Elvehjem (2) employing hypoxanthine as the substrate. The manometric data were substantiated, in the case of the tumor studies, by spectrophotometric analyses. A typical determination was carried out as follows: A 3 per cent tumor homogenate (30 mg. tissue, wet weight/ml 0.039 M potassium-sodium phosphate buffer, pH 7.4) was prepared by grinding the tissue in a glass homogenizer of the Potter type (10). The homogenates were centrifuged at 22,000 × *g* for 30 minutes at 0° C. One ml. of the resulting clear, supernatant fluid was added to each of six test tubes containing 2.5 ml. of 0.039 M potassium-sodium phosphate buffer, pH 7.4. The tube contents were aerated by bubbling compressed air, free of carbon dioxide and ammonia, through the mixture for 1 minute. This was followed by incubation in a water bath at 37° C. for 40 minutes to decompose endogenous purines. At the end of this initial incubation period, 0.3 ml. of a 1 mM xanthine solution (pH 7.4) was added to tubes 1 and 2, and 0.3 ml. of 0.039 M potassium-sodium phosphate buffer (pH 7.4) was added to tubes 3 and 4. These latter tubes serve as the enzyme blank. (No additions were made to tubes 5 and 6 until just prior to reading, at which time 0.3 ml. of 1 mM xanthine was added.) All six tubes were aerated as described above, and the incubation was continued for 1 hour. The extinction at 270 mμ (E_{270})[1] was then measured against the enzyme blanks (tubes 1 and 2) using a Beckman DU spectrophotometer. The difference in absorption between the tubes containing the enzyme plus the xanthine added after incubation (tubes 5 and 6) and the tubes in which the tissue extract and xanthine were incubated together (tubes 1 and 2) is taken to represent the xanthine decomposed by enzymatic action. An enzyme unit of xanthine oxidase is defined as that amount of enzyme which causes a decrease in the E_{270} of 0.001/hr at 37° C.

RESULTS AND DISCUSSION

Evidence is presented demonstrating that flavotin must be administered prior to 8-azaguanine

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† Dazian Foundation Fellow.

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[1] The protein content of the tissue extracts used, calculated according to the procedure of Kalckar (7), is of such a magnitude as to produce little interference in E_{270}, provided proper blanks are included.

in order to augment the carcinostatic action of this purine antagonist (Table 1). Simultaneous administration of these two compounds or the injection of 8-azaguanine prior to flavotin therapy was without potentiating effect. A similar phenomenon was observed in the case of 6-formylpteridine (15).

The lack of effect of *in vitro* addition of flavotin on mouse liver guanase and xanthine oxidase is presented in Table 2. These results tend to discount substrate antagonism as an explanation for the action of flavotin. 6-Formylpteridine, however, has been observed to inhibit guanase *in vitro*, irreversibly or "pseudo-irreversibly"[2] (4).

TABLE 1

EFFECT OF THE TIME RELATIONSHIP BETWEEN THE ADMINISTRATION OF FLAVOTIN AND 8-AZAGUANINE ON THE 755 CARCINOMA IN C57 FEMALE MICE*

Exper. no.	Group	Time interval† (hr.)	Mean tumor wt. ± 1 S.E. (mg.)	No. of animals dead/total	Per cent change in body wt.
72	Azaguanine		578 ± 73	0/19	+ 1
	Azaguanine+Flavotin	+1	272 ± 38	1/19	− 6
	"	simultaneous	568 ± 65	1/19	− 2
	"	$-\frac{1}{2}$	435 ± 62	0/19	− 3
101	Azaguanine		777 ± 99	1/19	+ 1
	Azaguanine+Flavotin	+1	292 ± 32	3/20	− 2
	"	simultaneous	657 ± 98	2/20	− 7
	"	$-\frac{1}{2}$	538 ± 74	2/20	− 7
104	Azaguanine		929 ± 112	0/20	+10
	Azaguanine+Flavotin	+1	365 ± 66	5/24	+ 5
	"	simultaneous	1184 ± 139	3/20	+ 9
	"	$-\frac{1}{2}$	687 ± 99	0/20	+ 4

* 8-Azaguanine injected at a dose of 50 mg/kg. Flavotin injected at a dose of 60 mg/kg.

† +Flavotin injected before 8-azaguanine.
−Flavotin injected after 8-azaguanine.

TABLE 2

EFFECT OF *in Vitro* ADDITION OF FLAVOTIN ON MOUSE LIVER GUANASE AND XANTHINE OXIDASE

Flavotin in reaction mixture (M/ml)	Guanase activity*	Xanthine oxidase activity†
0.000	910	103
0.011	922	118
0.023	910	106

* μg NH_3-N/gm tissue wet weight present after 2 hours incubation at 37° C.

† μl O_2 uptake/gm tissue wet weight/20 min at 37° C.
Values are the average of two experiments run in duplicate.

These observations, together with the earlier finding (13) that the carcinostatic enhancing action of flavotin could be reversed by riboflavin-5-phosphate, indicate that flavotin is probably antagonizing a flavo-enzyme which deals with purine metabolism, xanthine oxidase being the logical possibility. Such an explanation might also account for the temporal relationship involved in maximal activity. There are, in general, three ways in which a vitamin antagonist may produce its effects: by direct competition with the prosthetic group of the enzyme (8), inhibition of the enzyme system dealing with cofactor synthesis through substrate-antagonist competition (8), and the metabolic alteration of the inhibitor to form a substance which competes with the cofactor for the apoenzyme (17, 18). Of the three, only the first could produce a rapid effect, being limited only by the rate of dissociation between the prosthetic group and the apoenzyme.[3] However, even in this case, if the two compounds (flavotin and 8-azaguanine) were administered simultaneously, the optimum activity due to flavotin would have to be realized in 20–30 minutes, since it is during this period of time that almost all the 8-azaguanine administered is deaminated to the noncarcinostatic compound, 8-azaxanthine.[4]

In view of these considerations, xanthine oxidase was determined in tumor and liver tissues of animals from *in vivo* experiments similar to those reported above (Table 3). Measurable amounts of xanthine oxidase were found in tumors grown in animals not treated with flavotin. However, no xanthine oxidase activity was detected in tumors from animals which had received flavotin therapy. Liver tissues from the animals bearing these tumors had 8–10 times the xanthine oxidase ac-

[2] A "pseudo-irreversible" inhibitor as defined by Ackermann and Potter (1) is one which has such a small dissociation constant that it behaves like an irreversible inhibitor in its enzyme-titrating function. However, it can be dissociated from the enzyme with the result that enzymatic activity is restored.

[3] For purposes of simplification, complicating factors such as blood supply and tissue permeability have been omitted.

[4] Dr. J. Kream, private communication.

tivity observed in the tumors. A comparison of xanthine oxidase activity of flavotin-treated and nonflavotin-treated animals shows that flavotin had no appreciable effect on the xanthine oxidase activity of mouse liver tissue. Since the xanthine oxidase activity of the 755 tumor was of such a low magnitude that the majority of the total oxygen uptake consisted of endogenous respiration, it was essential that the manometric data be checked by an alternate method. The results of such analyses are presented in Table 4. Under the experimental conditions, xanthine was observed to disappear from the tumor extracts of flavotin-treated animals at a much slower rate than that in animals not treated with flavotin. These data, together with the manometric analyses, demonstrated beyond a reasonable doubt that flavotin antagonizes the flavo-enzyme, xanthine oxidase. The *in vivo* results presented in this paper and previously (13), together with the report of Shunk *et al.* (16) stating that flavotin administered alone has some activity against a lymphosarcoma grown in mice maintained on a riboflavin-deficient diet, demonstrate that flavotin is, at best, a weak riboflavin antagonist at the mammalian level.

We have observed (3) that the vitamin B_6 antagonist, desoxypyridoxine, antagonizes the glutamic-aspartic transaminase activity of normal mouse tissues to a degree inversely proportional to the concentration of glutamic-aspartic transaminase in the tissue involved—i.e., tissues of high activity, such as heart, are more resistant to desoxypyridoxine antagonism than tissues of low activity, such as testis and lung. Similar results have been reported by Reif and Potter (11) concerning the inhibition of succinoxidase by antimycin A. Similar behavior on the part of flavotin would explain the observed liver xanthine oxidase resistance and tumor xanthine oxidase susceptibility to flavotin antagonism. A closer examination of the data (Table 3) reveals that the liver xanthine oxidase activity of the flavotin-treated animals could be affected to the same numerical extent as the tumor xanthine oxidase and still go undetected. This is owing to the extremely high xanthine oxidase activity of liver and the usual high degree of biological variation as denoted by the standard error. In fact, such a trend does appear to exist. If this is the case, then a more active riboflavin antagonist or a higher dosage of flavotin should produce the results expected. Unfortunately, a test is impractical, since the flavotin is already being administered at the highest tolerated level.

Early in the course of these studies it was observed that guanase is susceptible to product inhibition (Table 5). Increasing levels of xanthine inhibited guanase in both a phosphate and a borate medium. That the borate and phosphate ions had no effect *per se* on guanase activity is demonstrated by the fact that no differences were observed in the absence of xanthine. Added xanthine was more effective in inhibiting guanase in the presence of borate than in a phosphate medium. It has been demonstrated (12) that the borate ion inhibits xanthine oxidase. Thus, in a phosphate medium, xanthine is being rapidly removed from the system. This lowers the amount of xanthine available to inhibit guanase while, in the case of the borate system, less xanthine is removed owing to inhibition of the xanthine oxidase

TABLE 3

EFFECT OF FLAVOTIN ADMINISTRATION ON XANTHINE OXIDASE ACTIVITY OF TUMOR AND LIVER*

	TUMOR	LIVER
	μl O_2/gm wet wt/20 min	μl O_2/gm wet wt/20 min
Saline	14±3 (20)	110±11 (7)
Flavotin†	0±2 (17)	100± 8 (7)

* 755 Adenocarcinoma grown in C57 female mice. Liver values are from the same tumor-bearing mice and include the standard error, $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$. The figures in parentheses represent the number of individual observations.

† 60 mg/kg.

TABLE 4

EFFECT OF FLAVOTIN ON TUMOR XANTHINE OXIDASE ACTIVITY*

	Units/gm wet wt†
Saline	3050 (6)
Flavotin (60 mg/kg)	300 (12)

* 755 Adenocarcinoma grown in C57 female mice. The figures in parentheses represent the number of individual observations.

† One unit is defined as that amount of xanthine oxidase that will give a decrease in E_{270} of 0.001/hr at 37° C.

TABLE 5

In Vitro INHIBITION OF MOUSE LIVER GUANASE BY XANTHINE*

XANTHINE IN REACTION MIXTURE (M/ml)	μG NH_2-N/GM TISSUE WET WT PRESENT AFTER 2 HR. INCUBATION WITH SUBSTRATE AT 37° C., pH 8.0†	
	0.1 M $PO_4^{\equiv}$	0.1 M Borate
0.000	731	718
0.005	717	608
0.011	617	535
0.016	608	408

* 8-Azaguanine was added at a level of 0.011 M/ml of reaction mixture.

† Corrected for endogenous ammonia. Values are the average of two experiments run in duplicate.

by the borate ion. These data are of prime importance in view of the effect of flavotin on tumor xanthine oxidase activity, since they demonstrate that an alteration in xanthine metabolism can in turn alter guanine metabolism. Hence, it is possible that, during the time that the xanthine oxidase in the tumor cell is inhibited by flavotin, endogenous xanthine accumulates at a rate faster than it is diffused through the cell membrane. 8-Azaguanine introduced into such a local environment at this time would be deaminated at a slower rate than would occur in a noninhibited system due to the high local concentration of xanthine (Chart 1). The effect would be the same as removing guanase from the tumor or increasing the dosage of 8-azaguanine, thereby producing a greater carcinostatic effect.

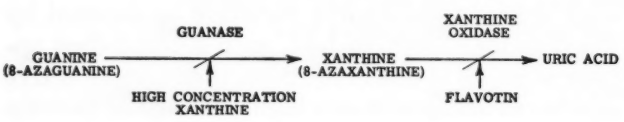

CHART 1.—Postulated mechanism whereby flavotin potentiates 8-azaguanine carcinostasis.

SUMMARY

The ability of flavotin to enhance the carcinostatic action of 8-azaguanine on the 755 tumor grown in C57 female mice was found to be dependent on a temporal relationship. Positive results were obtained only if the 8-azaguanine was injected 1 hour after the administration of flavotin. Simultaneous treatment or the administration of 8-azaguanine prior to flavotin was ineffective.

The *in vitro* addition of flavotin had no effect on the activity of guanase or xanthine oxidase in mouse liver.

Xanthine oxidase activity was detected in the 755 tumor. The *in vivo* administration of flavotin was observed to antagonize the tumor xanthine oxidase activity. Under the same conditions, flavotin had no measurable effect on liver xanthine oxidase.

It was demonstrated that guanase is susceptible to product inhibition.

It was postulated that flavotin potentiates the carcinostatic action of 8-azaguanine by inhibiting tumor xanthine oxidase, thereby indirectly inhibiting guanase through product inhibition. Guanase inhibition would prevent the deamination of the carcinostatic compound, 8-azaguanine, into the noncarcinostatic compound, 8-azaxanthine, thus increasing the efficacy of 8-azaguanine as a carcinostatic agent.

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Quantitative Biochemical Differences between Tumor and Host as a Basis for Cancer Chemotherapy I. Vitamin B_6*

DANIEL M. SHAPIRO,† MAURICE E. SHILS, AND L. S. DIETRICH

WITH THE TECHNICAL ASSISTANCE OF A. ATTI, E. BORRIES, A. FINE, R. FUGMANN, P. HAYWORTH, AND E. ZANAR

(Department of Surgery, College of Physicians and Surgeons, School of Public Health, and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y.)

It has been postulated that cancer chemotherapy utilizing metabolic antagonists may be based on quantitative differences between normal and tumor tissue. In general, neoplasms have considerably lower enzyme and vitamin concentrations than most normal tissues, and, in some cases, the observed levels are lower in the cancer cell than in any normal tissue. Furthermore, it is known that inhibition by an antimetabolite depends upon the concentration ratio of the metabolite, or susceptible enzyme, and antagonist. Thus, it is reasoned that an enzyme in low concentration in cancer tissue may be blocked by means of an antagonist, while the activity of the same enzyme in nonmalignant cells would be only minimally depressed owing to its higher concentration.

This concept has been previously recorded. Boyland (6) selected a catalase inhibitor, α-nitroso-β-naphthosol for chemotherapeutic trial, reasoning that the significantly lower enzyme concentration in certain tumor tissues should permit inhibition of tumor growth with doses of this agent tolerated by normal tissues. Ackermann and Potter (1) stated that ". . . it should be possible to completely inhibit an enzyme present in cancer tissue in small amounts, while producing only partial inactivation of the enzyme in tissues containing it in larger amounts." Noting that pyridoxine had been reported to be in low concentration in most solid tumors studied (16), Shapiro and Gellhorn (21) tested desoxypyridoxine, a vitamin B_6 antagonist for mammals (8, 26), and found it to be carcinostatic against the 755 mouse mammary carcinoma. Ayengar and Roberts (5) have also suggested this hypothesis on the basis of their finding that ". . . glutamine levels in the tumors were uniformly lower than in most of the normal tissues."

The chemotherapeutic approach suggested requires further detailed appraisal. This report attempts to accomplish this by co-ordinated microbiological, biochemical, and biological studies of pyridoxine-desoxypyridoxine relationships in a single tumor-host system.

MATERIALS AND METHODS

General.—C57BL mice of both sexes, 2–4 months of age and weighing 18–25 gm., were maintained in plastic cages in an air-conditioned constant-temperature room (74° F.) and received ad libitum a diet of Rockland pellets and water. The neoplasm employed in this study was the 755 tumor, a mammary adenocarcinoma, transplanted into the axillary region by the usual trocar technic. Testosterone in sesame oil was injected intramuscularly, and desoxypyridoxine, dissolved in saline, was administered intraperitoneally. Daily therapy was initiated at various intervals after transplantation, as indicated in the tabular summaries, and continued until the experiment was completed. Dosage is stated in the tables.

Vitamin B_6 analysis.—Tissues were removed and weighed on a micro-torsion balance immediately after the mice were killed by decapitation. The tissues were then homogenized in about 0.5 ml. of 0.055 N HCl in a glass homogenizer of the Potter-Elvehjem type, transferred to volumetric flasks of the appropriate size and the volumes adjusted with 0.055 N HCl. Aliquots were placed in amber bottles and autoclaved at 20 pounds pressure for 5 hours, cooled, adjusted to pH 5.0, diluted to convenient volume and stored at 5° C., or in some experiments at −20° C., until analyzed.

The assay procedure was that of Atkin, Schultz, Williams, and Frey (4) with the following two modifications in the basal medium: the casein hydrolysate was prepared according to the method of Rubin, Scheiner, and Hirschberg (18), and niacin was added as recommended by Hopkins and Pennington (13). Each organ or tissue was analyzed individually to ascertain the pattern and uniformity of the vitamin concentration within the individual and among individuals.

The percentage dry weights were found for each tissue by

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drying the tissues to constant weight in an oven at 102° C. Data calculated on a dry weight basis showed essentially the same trend as on a wet weight basis and, therefore, are not reported.

Statistical evaluation between groups was made employing Fisher's *t* test.

Glutamic-aspartic transaminase studies.—The animals were killed by cervical rupture, and the tissues were immediately removed and chilled in ice-cold isotonic saline. Glutamic-aspartic transaminase activities were measured according to the colorimetric procedure of Tonhazy *et al.* (25) and are reported in Q_T^{10} units as defined by Ames and Elvehjem (2), i.e., μl of CO_2 (pyruvate) evolved/hour/mg tissue dry weight for the incubation time indicated. All animals received their last injection of desoxypyridoxine 1 hour prior to being sacrificed. The final dose of testosterone was administered the preceding day.

Biological.—8-Azaguanine, in weakly alkaline solution, was administered daily by intraperitoneal injection and was included in all experiments to provide base-line data for future combination chemotherapy experiments. Its consistent inhibitory effect upon the 755 tumor, the details of tumor growth measurement, and the method of statistical analysis have been previously described (19–21).

RESULTS

Vitamin B_6 concentration.—Data on the total concentration of vitamin B_6 in the 755 tumor in relation to normal tissues in individual male mice are summarized in Table 1. It will be noted that the vitamin concentration is lower in the tumor than in any normal tissue except spleen and lung, and, occasionally, testis. Because, in the case of the spleen, there may be large and variable amounts of blood and leukocytes, the data for this organ undoubtedly do not accurately reflect the parenchymal content.

In view of Stoerk's report that testosterone prolonged the survival of pyridoxine-deficient rats (23), Cohen and Hekhuis's suggestion of an inverse relationship between transamination and the synthesis of protein (7) and Hertz's work showing "... the essentiality of pyridoxine for the response to androgens ..." (14), it appeared worth while to investigate the relationship of testosterone to pyridoxine metabolism. Experiments were conducted on the effect of testosterone administration upon the vitamin B_6 content of the 755 tumor. The results, which are summarized in Table 2, reveal that testosterone therapy significantly lowered the vitamin B_6 levels in the tumors.

Glutamic-aspartic transaminase studies.—The inhibitory effect of desoxypyridoxine upon the glutamic-aspartic transaminase activity of various C57 mouse tissues is demonstrated in Table 3. Inhibition of this vitamin B_6-containing enzyme was progressive up to the 8–12-day period when the maximum effect was reached. Increasing the level of desoxypyridoxine given and/or lengthening the period of desoxypyridoxine therapy had little influence on transaminase activity once the point of maximum inhibition had been reached. As recorded graphically in Chart 1, the degree of

TABLE 1

COMPARISON OF THE VITAMIN B_6 CONTENT OF NORMAL MALE C57 MOUSE TISSUES AND THE 755 TUMOR*

(μg/gm wet weight)

♂ Mouse	Tumor	Liver	Kidney	Heart	Lungs	Brain	Testes	Muscle	Stomach	Spleen
1	1.36	5.99	4.64	4.85	1.06	2.73				
2	0.80	6.04	4.20	4.16	1.06	2.06	1.07	2.17	1.89	0.97
3	0.73	6.33	3.88	7.00	0.67	3.12	0.89	3.10	1.19	0.50
4	1.01	11.46	12.32	12.20	0.98	5.15	2.25	2.56	1.33	0.53
5	0.96	5.18	7.38	5.58		3.03	1.49	4.16	1.67	1.96
8	0.39	4.45	2.33	2.86		2.88	1.49	2.74	1.82	1.71
9	1.12	4.62	2.57	3.32	0.94	2.41	0.81	2.85	1.79	0.81
10	0.37	3.2	1.97	3.12	0.76	1.86	0.70	1.26	0.87	0.58
11	0.29	2.9	2.73	2.31	0.59	2.37	1.01	2.67	1.50	0.89
12	0.59	4.37	2.98	3.99	0.75	3.41	0.83	3.87	1.85	0.55
14	0.36	3.59	2.04	2.55	0.48	2.13	0.48	2.45	1.40	0.71
15	0.33		2.87	3.54	0.48	2.93	0.60	1.90	1.17	0.50
Mean	0.69	5.28	4.16	4.62	0.78	2.84	1.06	2.70	1.50	0.88
Sigma (σ_m)	0.104	0.709	0.859	0.791	0.071	0.246	0.154	0.248	0.010	0.151

* Tissue extracts kept in frozen state prior to analysis.

TABLE 2

EFFECT OF TESTOSTERONE ON THE VITAMIN B_6 CONTENT OF THE 755 TUMOR IN FEMALE C57 MICE*

Testosterone Treated		Controls	
	B_6 (μg/gm)		B_6 (μg/gm)
No. mice	Mean±S.E.	No. mice	Mean±S.E.
9	0.262±0.013	9	0.423±0.060

* On wet weight basis.

Testosterone was dissolved in sesame oil and administered intramuscularly at 50 mg/kg, daily therapy beginning 1 day after transplantation of tumor. Controls received daily intramuscular injections of sesame oil in comparable volume to treated mice. Duration of tumor growth was 21 days. The difference between the two means is statistically significant at the 2 per cent level.

inhibition was inversely proportional to the concentration of transaminase in the tissues.

Biological studies.—In vivo data have been obtained demonstrating the ability of testosterone to bring forth the carcinostatic properties of very low doses of desoxypyridoxine (Table 4). Although high doses of desoxypyridoxine have carcinostatic

4. Study of a vitamin B_6-containing enzyme, glutamic-aspartic transaminase, reveals it to be present in low concentration in the tumor and to be inhibited by desoxypyridoxine.

5. In general, the lower the initial enzyme concentration in a tissue the greater the degree of inhibition by an antagonist to this enzyme.

TABLE 3

EFFECT OF DESOXYPYRIDOXINE ON THE GLUTAMIC-ASPARTIC TRANSAMINASE ACTIVITY OF VARIOUS TISSUES OF THE MOUSE

Exp. no.	Days on exp.	Testes Q_T^{10}	Lung Q_T^{10}	Liver Q_T^{10}	Kidney Q_T^{10}	Heart Q_T^{10}	Tumor Q_T^{10}
1	0	116± 5	157±13	350±15	313±16	628± 8	
	4	127± 8	114±12	220±12	229± 6	596±14	
	8	78±12	71± 6	194± 7	190± 6	594±25	
	12	0± 0	45± 8	142± 9	109±17	488±10	
	16	24±10	74± 7	153± 6	143±17	478± 5	
2	0	118±59	116±11	304±23	272±29	656±78	
	12	27±13	41± 7	138±56	171±29	520±52	
	19	36±19	22±18	128±26	123±33	510±78	
3	0						192±6
	21						51±8

Adult C57 male mice on a stock diet containing ample vitamin. Desoxypyridoxine was administered intraperitoneally at a level of 150 mg/kg/day in experiments 1 and 3, and at 175 mg/kg/day in experiment 2. Animals were sacrificed 1 hour after the last injection. Values are the average of eight observations/group and include the standard error, $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$. Nontumor-bearing mice were used in experiments 1 and 2.

activity against this tumor (21), a dose too low to affect tumor growth was chosen in order to indicate clearly the relationship between low vitamin B_6 levels in the tumor and carcinostatic effect. An earlier publication (19) has already documented the lack of carcinostatic effect of testosterone, alone or in combination with 8-azaguanine, on the 755 tumor. As can be seen from Table 4, with the one exception of Exp. 118, the triple combination of 8-azaguanine plus testosterone plus desoxypyridoxine consistently inhibited tumor growth to a significantly greater extent than 8-azaguanine alone or any double combination.

DISCUSSION

These data offer evidence in favor of the hypothesis that low metabolite concentrations render malignant cells vulnerable to antagonist chemotherapy:

1. The 755 tumor is a neoplasm in which vitamin B_6 is in lower concentration than in the majority of normal tissues of its host.

2. Therapeutic doses of the vitamin B_6 antagonist, desoxypyridoxine, have carcinostatic activity against the 755 tumor (21).

3. Testosterone decreases the vitamin B_6 concentration of the tumor, and this may be the mechanism by which testosterone increases the effectiveness of a given dose of desoxypyridoxine.

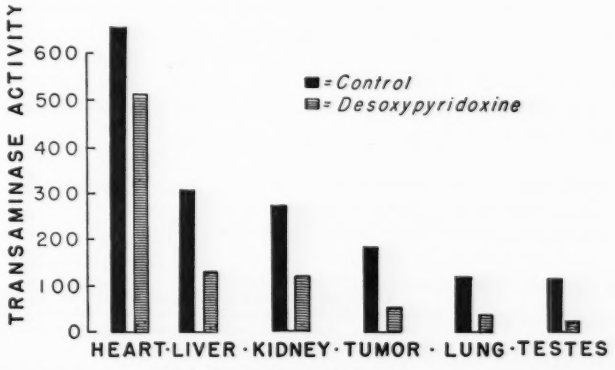

CHART 1.—Desoxypyridoxine antagonism of glutamic-aspartic transaminase activity. Tissue values are expressed in Q_T^{10} units and are from male C57 mice fed a stock diet ad libitum. All animals received desoxypyridoxine at a level of 175 mg/kg/day via intraperitoneal injection, for a 19-day period. The tumor is the 755 mammary adenocarcinoma grown in male C57 mice under similar conditions. The tissue values are the average of eight individual observations.

The slight mortality in the groups of mice receiving the desoxypyridoxine without testosterone may be due to the fact that daily therapy was given on an individual body weight basis rather than on an individual blood or tissue level basis. It can be noted in Table 1 that the vitamin B_6 values in the normal tissues cover a fairly wide range, e.g., from 1.86 to 5.15 μg/gm in brain tissue. The relative lack of toxicity in the experimental groups

receiving desoxypyridoxine without testosterone may be explainable on the basis that the dose of desoxypyridoxine employed does not reach the "inhibition index" for even those tissues whose vitamin B_6 levels are at the lower concentrations. The increased mortality seen in the groups receiving the testosterone-desoxypyridoxine combination may be due to reduction of tissue levels of vitamin B_6 to still lower concentrations at which desoxypyridoxine is extremely effective. It seems likely that basing daily dosage on individual blood or tissue levels would prevent the administration of a lethal dose of the antagonist and yet permit a carcinostatic effect.

Additional vitamins and enzyme systems will have to be similarly documented in the 755 and other tumors before the hypothesis can be considered to have a firm foundation and general applicability in the field of cancer chemotherapy. However, the probability that the hypothesis is a promising lead may already be found in the publications of other investigators. The concentration of riboflavin in cancer tissue has been found to be lower than that in normal tissue (16). Aposhian and Lambooy (3) have found diethylriboflavin, a riboflavin antagonist, to possess carcinostatic properties. Another riboflavin analog, 6-chloro-9-(1′-D-sorbityl)-isoalloxazine, or flavotin, potentiates 8-azaguanine carcinostasis on the 755 tumor in female mice (20) by blocking the low tumor concentration of the flavo-enzyme, xanthine oxidase (9). Investigators at the National Cancer Institute have reported that cytochrome oxidase is present in low amounts in most tumors (11) and that a-peltatin, a potent antagonist of this enzyme, possesses carcinostatic properties (10, 15, 27). Hirschberg *et al.* (12) have demonstrated a correlation between low tumor guanase activity and tumor susceptibility to 8-azaguanine. The low concentration of tumor guanase makes possible augmentation of the carcinostatic activity of 8-azaguanine by tolerated doses of 6-formylpteridine, a guanase inhibitor (22). Using antimycin A as an inhibitor of the succinoxidase system, Reif and Potter (17) have shown that, in general, the degree of enzyme antagonism in a tissue is inversely proportional to enzyme concentration of the tissue.

Although the aforementioned publications all

TABLE 4

POTENTIATING EFFECT OF TESTOSTERONE ON A NONCARCINOSTATIC DOSE OF DESOXYPYRIDOXINE*

Exp. no.	Sex	Group†	Mean tumor wt. ±1 S.E. (mg.)	No. animals dead/total	Per cent change in body weight
113	M	Saline Controls	2942±260	0/19	+14
		Aza.	603± 49	0/20	+ 6
		Aza.+Test.	497± 77	0/19	+14
		Aza.+Des.+Ses. oil	607± 77	1/20	+ 6
		Aza.+Des.+Test.	288± 58	4/20	+12
97	M	Aza.	515± 56	0/20	+ 4
		Aza.+Test.	374± 33	1/20	+ 9
		Aza.+Des.	404± 73	2/20	− 3
		Aza.+Des.+Test.	131± 21	4/20	+ 4
106	M	Aza.+Test.	243± 56	0/19	+20
		Aza.+Des.+Test.	82± 13	2/19	+12
110	M	Aza.	538± 41	1/19	+ 6
		Aza.+Des.	398± 44	0/20	+ 4
		Aza.+Des.+Test.	146± 25	2/21	+ 8
116	M	Aza.	754± 88	2/20	+ 7
		Aza.+Test.	594± 74	0/20	+18
		Aza.+Des.	1227±118	3/20	+ 8
		Aza.+Des.+Test.	224± 52	4/20	+ 9
117	F	Aza.	693±105	0/19	+ 3
		Aza.+Des.	1072±112	2/20	+ 4
		Aza.+Des.+Test.	353± 66	1/20	+14
118	F	Aza.	945±122	0/18	+ 6
		Aza.+Des.	1083±131	1/20	0
		Aza.+Des.+Test.	764±105	3/18	+12

* Desoxypyridoxine was administered intraperitoneally at a dose of 40 mg/kg twice daily, 4 hours apart. Testosterone was dissolved in sesame oil and administered intramuscularly at a dose of 25 mg/kg once daily with the first injection of desoxypyridoxine. 8-Azaguanine was administered intraperitoneally at a dose of 50 mg/kg once daily with the second dose of desoxypyridoxine.

† In all experiments therapy was begun upon well established (7 days after transplantation) 755 mammary adenocarcinomas grown in C57BL mice. Duration of tumor growth varied between experiments from 24 to 34 days.

Abbreviations: S.E. = Standard Error; Aza. = 8-Azaguanine; Test. = Testosterone; Ses. oil = Sesame oil; Des. = Desoxypyridoxine.

support the thesis that low metabolite concentrations in a tumor afford a basis for chemotherapy, there are proponents for the idea of utilizing high concentrations as a guide for the selection of chemotherapeutic agents. Swendseid *et al.* (24) found the folic acid content of the leukocytes to be higher in leukemic than in normal subjects and stated that this rendered the leukemic cells ". . . more susceptible to the action of folic acid antagonists. This explanation provides a rational basis for the use of the folic acid antagonists in the treatment of the acute leukemias." However, there are a number of peculiarities about the folic acid system that must be considered. It is generally agreed that conjugated folic acid derivatives per se are metabolically inactive, and the folic acid group appears to be unique among vitamins of the B complex in that certain tissues can store conjugated forms of this vitamin. Thus, the total folic acid content of a tissue may not give a true picture of the concentration of folic acid-containing enzyme systems, which are undoubtedly the important point of attack for folic acid antagonists. Therefore, the high concentration of total folic acid in leukemic cells does not necessarily mean that this is why folic acid antagonists are successful in alleviating this disease. More detailed information about folic acid-containing enzymes may eventually reveal that these enzymes are present in lower concentration in leukemic leukocytes than in normal. Finally, Pollack and coworkers (16) have found that most neoplasms have much higher amounts of total folic acid than do normal tissues. In view of these observations, if high amounts of folic acid render tissues susceptible to the action of folic acid antagonists, it is of interest that the majority of neoplasms are not particularly sensitive to such therapy.

Finally, the *in vivo* results obtained here emphasize the possibility that the utilization of combinations of chemical agents to damage simultaneously multiple metabolic pathways in tumor cells may be practical. Combination chemotherapy may also offer the prospect of attacking a cancer cell's metabolism at sufficiently diverse points to result in death of the cell before the development of drug resistance or drug dependence.

SUMMARY

A mouse mammary carcinoma (755), previously demonstrated to be sensitive to desoxypyridoxine therapy, has a lower concentration of vitamin B_6 than the majority of the normal tissues of the host animal. A vitamin B_6-containing enzyme, glutamic-aspartic transaminase, is also present at a low concentration in the same tumor and is inhibited by desoxypyridoxine. This inhibition of transaminase in both tumor and normal tissues is inversely proportional to enzyme concentration. The vitamin B_6 level in the tumor is significantly decreased by testosterone therapy, although such treatment has no effect on tumor growth. Combination chemotherapy with testosterone and desoxypyridoxine results in inhibition of tumor growth with doses of the pyridoxine antagonist that are without carcinostatic effect when administered alone.

The data and pertinent literature are discussed as evidence for the hypothesis that the low concentrations of metabolites in neoplastic tissue furnish a basis for the selection of chemotherapeutic agents.

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Lack of Effect of High Potency Arginase on Tumor Growth*

DAVID M. GREENBERG AND E. NORBERG SASSENRATH

(Department of Physiological Chemistry, University of California School of Medicine, Berkeley, Calif.)

The amino acid arginine and the enzyme arginase, which catalyzes the hydrolysis of the amino acid to ornithine and urea, have been alleged to have a special position in relation to the neoplastic process (4). This subject is now of more than mere experimental interest, because for several years cancer victims have been sold courses of treatment with arginase-containing liver preparations.

The rationale for the linking of arginase to the neoplastic process is based on the following experimental evidence:

1. Early investigations appeared to indicate that cancerous tissue contained an unusually high content of arginine (13). This has not been borne out by more recent analytical studies (2, 3, 19). In fact it now appears to be well established that the amino acid composition of most tumors is very much like that of most other animal tissues.

2. Arginine has been reported to be effective in increasing the rate of growth (8) and mitosis (1) of tumors. However, inhibition of tumor growth by arginine has also been reported (14). In the experiments reported below, no noticeable effect was found in either direction. Bach and Lasnitzki (1) observed that arginine increased the rate of mitosis in tissue cultures of a mouse adenocarcinoma, but not in similar cultures of normal embryonic mouse lung. A similar increase in mitosis was produced by ornithine, which has the property of inhibiting the activity of arginase.

3. There are marked differences in the arginase activity of cancerous tissue from that of comparable normal tissue. The published data show that the arginase activities of tumors of diverse origin tend toward a common level which is below the activity of organs high in arginase content (liver, kidney) and is markedly higher than that of the rest of the organs and tissues of the body (11). Examples of the latter are the increased arginase activity observed with onset of malignancy in methylcholanthrene-induced epidermal carcinoma in mice (18) and in spontaneous mammary carcinoma (11) over the levels in normal epidermis or in the hyperplastic breast. This trend of arginase to a common level of activity in tumors is in harmony with numerous other observed biochemical changes in neoplasms (10). Greenstein and coworkers have pointed out the difficulties in assessing the significances of changes in enzyme activity in relation to the neoplastic change.

The appearance of arginase in muscle and other tissues of cancer-bearing mice has been reported to occur even while the tumors were quite small, while almost none is present in these tissues in normal animals (13). On the other hand, the arginase activity of kidney and liver was depressed in the tumor-bearing host (7). Bach and Lasnitzki (1) observed in experiments with a carcinoma that slowly growing tumors contained twice as much arginase as rapidly growing tissue. From this they concluded that arginase contributed a defense mechanism against tumor growth by inhibiting its growth rate.

It logically follows from this hypothesis that administered arginase could exert a carcinostatic effect. Since arginine is an essential constituent of tissue protein and arginase destroys this amino acid, a preferential inhibitory action on tumor growth might result if the injected arginase was preferentially accumulated in an active state by tumors, or if the arginine requirement of the tumor was greatly in excess of the amount compatible with the needs of the organism for existence.

A considerable number of reports have appeared on an inhibition of tumor growth produced by the administration of arginase preparations. A doubtful inhibition of tumor growth was indicated by Wiswell (25) on a mammary carcinoma implanted in C3H mice. Inhibition of growth was observed on the injection of arginase into mice implanted with an adenocarcinoma (17). Most remarkable results were claimed by Vrat (22–24), who reported complete regression, with striking

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histological changes and no recurrence of the tumors after cessation of the arginase treatment. Only Neukomm *et al.* (17) employed arginase of high purity.[1]

In view of the above claims, it appeared desirable to test the effects of an arginase preparation of high potency prepared in our laboratory (9) and also to study the disposition of injected arginase and its effect on the arginine levels of blood plasma, tumor, and liver. The latter tissue was studied because it is the main source of arginase in the body.

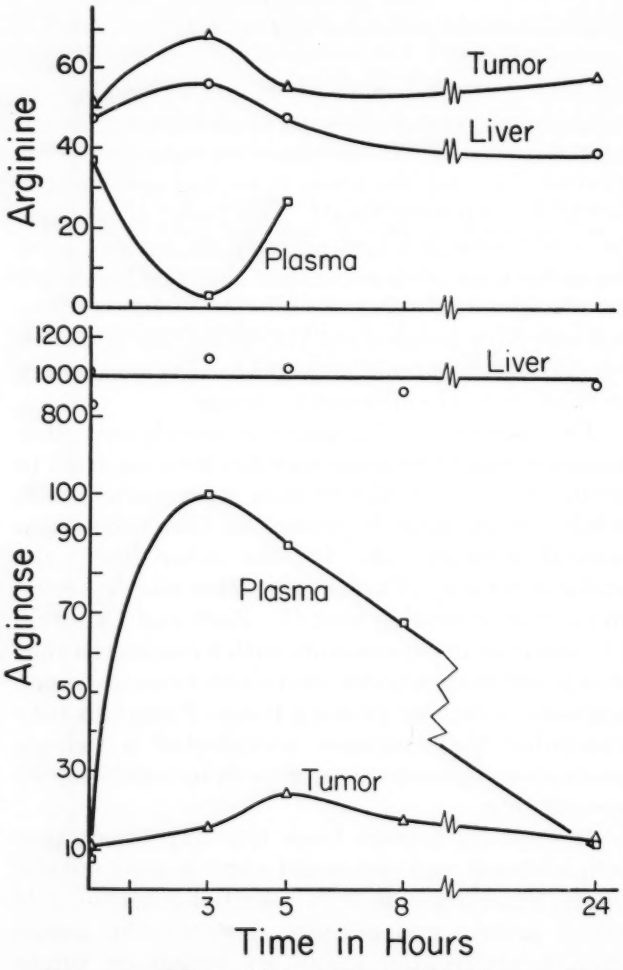

CHART 1.—Effect of intraperitoneal injection of 200 arginase units per mouse of manganese-activated arginase on the free arginine and arginase contents of plasma, liver, and tumor in C3H male mice bearing lymphosarcoma-6C3H-ED. Values are for pooled samples from triplicate animals. Arginase expressed in units and arginine in μg/gm fresh tissue.

MATERIALS AND METHODS

Animals and tumors.—The strain C3H mice used were 3–4-month-old males weighing 24–29 gm. Strain A female mice weighed 17–21 gm.; strain A males, 19–25 gm.

[1] Prepared by C. B. Thompson according to the procedure given in reference (20).

The tumors used with the C3H mice included the Shimkin mammary carcinoma (MC-S); the Gardner lymphosarcoma 6C3H-ED (LS-G); and the Ehrlich ascites tumor (Asc-E). The strain A mice bore a first transplant of a spontaneous mammary carcinoma; the tumor and mice were obtained from the same source as those used by Vrat.[2] The solid tumors were transplanted unilaterally by the trocar technic; the ascites tumor was transplanted by intraperitoneal injection of 0.2 ml. fresh ascitic fluid.

Injections.—Injections of the animals bearing solid tumors were started after the tumor transplants were sufficiently established to allow selection of experimental groups comparable in average body weight and tumor size, i.e., 5–9 days after transplanting. The periods of treatment were determined by the state of health of the animals; an attempt was made to avoid symptoms of cachexia during the later stages of tumor growth. Ehrlich ascites-bearing animals were injected during the 7 days following tumor transplantation.

Assay procedures.—Arginase activity was determined by a procedure combining the conditions of incubation of Van Slyke and Archibald (21) with the colorimetric determination of urea by Engel and Engel (5, 9). The incubation was carried out at 25° C. and pH 9.5 in 0.285 M arginine. The urea formed was precipitated as dixanthyl urea, which was determined colorimetrically in 50 per cent H_2SO_4.

The assay procedure for all arginase preparations was standardized at pH 9.5 for uniformity. For Co^{++}-activated preparations, the activity at pH 7.0 assayed two-thirds the activity at pH 9.5. The Mn^{++}-activated preparations showed negligible assay activity at pH 7.0, but apparently were physiologically active (Chart 1).

Arginine determinations were made according to the microbiological assay procedures of Henderson *et al.* (11) using *Lactobacillus delbrueckii.*[3]

Solutions.—The Mn^{++}-activated arginase (Mn^{++}-arginase) employed in the tests was horse liver arginase prepared according to the procedure of Roholt and Bagot, described by Greenberg (9) and assayed 3000–3500 arginase units/mg nitrogen.[4] This was dissolved in a manganese buffer

[2] This tumor and the mice employed in the experiments were kindly furnished by Dr. Kenneth B. De Ome, Cancer Research Genetics Laboratory, University of California, Berkeley.

[3] We are indebted to Mrs. Lore S. Keffer for performing the microbiological assays of arginine.

[4] This is equivalent to an activity of 480,000–560,000 arginase units/gm protein. For purposes of comparison it may be pointed out that Thompson's arginase (20) employed in the experiments of Neukomm *et al.* (17) was reported to have an

solution at pH 7.5 which was 0.01 M in maleate and 0.05 M in $MnSO_4$ (9). The Co^{++}-activated arginase-1 (Co^{++}-Mn^{++}-arginase) was a less pure preparation similar to the above prepared by Roholt and Bagot. It was further activated by heating at 40° C. for 3 hours in 1 per cent sodium acetate containing 0.125 mg. Co^{++}/ml; it was then dialyzed against the same sodium acetate-Co^{++} solution.

Two Co^{++}-activated arginase samples were prepared by Dr. M. S. Mohamed.[5] One, Co^{++}-activated arginase-2, was prepared from horse liver according to the extraction procedure described by him in the presence of manganous ions (15). It was then converted to Co^{++}-arginase according to the procedure described above. A second Co^{++}-arginase was prepared by the same procedure (15) without access to manganous ions during its preparation. It was finally activated by dissolving and heating at 40° C. in 1 per cent sodium acetate, containing 0.125 mg. Co^{++}/ml.

The arginase preparation from the Permanente laboratory was supplied in phosphate buffer solution.[6] The solution was assayed as received and administered according to the procedure recommended—i.e., injected subcutaneously twice daily.

Crystalline bovine serum albumin (BSA), Armour, was injected to test the possibility of foreign protein reaction. In experiment VIII, the BSA and the arginase solutions had equal nitrogen contents.

Arginine in buffer was injected to test the possibility of enhancement of tumor growth under our conditions.

In each separate experiment listed in Table 1, each compound injected was dissolved in the same solvent or buffer used for the arginase—i.e., Mn^{++}-maleate buffer at pH 7.5, or 1 per cent sodium acetate plus cobalt acetate at pH 7.0. Most experiments have both a buffer-injected and a non-injected control group.

Units of arginase activity.—Arginase activity is expressed here in the units of Van Slyke and Archibald (21), assuming a zero order reaction and less than 7 per cent hydrolysis, where A.U. = μM urea liberated/minute at 25° C., pH 9.5, and 0.285 M substrate concentration.

This unit is approximately 0.4 that used previously in the literature by Mohamed and Greenberg (16), Neukomm *et al.* (17), Vrat (22–24), and Wiswell (25), which equates A.U. to a first order reaction constant under the conditions of 10–75 per cent hydrolysis at 40° C., pH 8.5, and 0.005 M substrate concentration. However, this difference between arginase unit values is apparently nearly all due to a temperature effect, since we find that assay activity increases 2.1 times between 25° and 40° C. with any arginase preparation. Thus, arginase of the same unit value by either definition will have nearly the same degree of enzyme activity at body temperature.

TABLE 1

ARGINASE CONTENT OF TISSUES OF NORMAL AND TUMOR-BEARING C3H MICE

			A.U./GM FRESH TISSUE		
ANIMAL	SEX	No.	Plasma	Muscle	Liver
Normal	F	5	2.1±0.48*	9.1±0.46	728± 73
Tumor-bearing†	F	4	1.7±0.40	8.8±0.35	756± 46
Normal	M	5	1.7±0.43	6.4±1.44	760±100
Tumor-bearing†	M	5	1.7±0.22	8.1±1.7	802± 28

* Mean deviation.

† Gardner lymphosarcoma; animals used 11 days after inoculation.

RESULTS

Effect of tumor on arginase activity of host.—The effect of the presence of tumors on the arginase activity of a number of tissues was tested in C3H mice, in view of the long assay incubation period of 24 hours employed by previous workers (4, 13). The results are recorded in Table 1. With the strain of mouse and the tumor employed for these tests no difference was found in the tissues from the normal and tumor-bearing animals. This is contrary to the results of Klein and Ziese (13). With this strain of mice no difference was found in activity between males and females, although a higher content of arginase in the liver of the males has been reported for many mammalian species.

Effects of injections of arginase on arginine and arginase.—Intraperitoneal injection of arginase

activity of 65,000 arginase units/gm protein. In a private communication Thompson has informed us that this figure is in error, and the actual activity was 40,500 arginase units/gm.

[5] We are greatly indebted to Dr. Mohamed for making the arginase preparations for us. The work was performed in our laboratory during the period when Dr. Mohamed was a Fulbright Fellow from Egypt.

[6] Several preparations of this arginase, activated with cobalt, were kindly furnished to us by Dr. Eaton McKay, Director of Research, Permanente Foundation, Oakland, California. They were prepared in the same manner as those used by Vrat. Enzyme assays on them were performed in our laboratory.

into normal and cancer-bearing mice caused considerable changes in the arginine content and arginase activity of the blood plasma and much lesser changes in the tumors or liver. In three groups of normal mice given 125 A.U., the plasma arginine level dropped in 3 hours to an average value of one-half normal. The arginase activity increased almost tenfold along a curve that mirrored the change in arginine; both returned virtually to normal in 8 hours. In the cancer-bearing animals 200 A.U. decreased the plasma arginine to one-seventh the original values, and the arginase activity increased about tenfold, the maximum effect occurring at 3 hours and returning to normal in 24 hours (Chart 1). Tumor and liver contents of arginine and arginase were altered comparatively little by the arginase injection (Chart 1).

Effect of arginase on tumor growth.—The effects of arginase administration on the growth of a series of tumors are recorded in Table 9. Average values are reported for total changes in the weights of the mice and tumors during the course of injection. Errors are the standard errors of the means, employing equations for small samples where $N < 30$.[7]

A statistical comparison of each treated group with its corresponding buffer control was carried out by calculation of the standard errors of the differences between means for small samples.[7] Observed differences were then compared to those calculated to be significant, using the t factors at $P = 0.1$ (6). This high level of significance was selected to allow any possible effects to be detected.

However, on the basis of this test, only those values italicized in the table appeared to be significant when compared to their respective controls. In addition, the results with the lymphosarcoma and ascites tumors indicate an apparent enhancing effect of arginase on tumor growth. An apparently significant inhibition of tumor growth with one of Vrat's preparations was accompanied by a significant inhibition of weight gain by the animal. Thus, this cannot be interpreted as a selective action on the tumor.

It is realized by the authors that extensive statistical treatment is not warranted for samples of such small size. The observations of apparently significant opposite effects on tumor growth by arginase serve to emphasize this. However, it seems reasonable to say that the results of the statistical treatment indicate the lack of any trend toward tumor inhibition in the experiments reported.

DISCUSSION

The results of the experimental work reported here lend no support to the belief that arginase could be particularly effective as a carcinostatic agent. The published experimental evidence on which this belief is based, except that of Vrat (22–24), is, to say the least, anything but impressive. The magnitude of reduction in mitosis by arginase, the rates of reduction of tumor growth reported by most investigators (1, 17, 25), and the reported increase in tumor size caused by arginine injection (8) usually are of a low order of magnitude, and, in fact, have not always been capable of confirmation by other investigators. Our own results in Experiment III show almost a 20 per cent increase in tumor growth in animals receiving injections of arginine, but this difference is not statistically significant.

The results of Vrat are unusual in that he has reported complete regressions without recurrence of spontaneous tumors in Swiss mice,[8] and of transplanted mammary carcinomas in strain A[9] and C3H mice upon administration of arginase. According to Vrat, all the treated tumors were considerably reduced in size, became firm and round, were encapsulated by connective tissue, and differed in staining properties from the control tumors. This difference was in the appearance of the chromatin material, and the cordlike arrangement of the cells was changed. Von Kossa stains indicated heavy calcium deposits in the cellular and extracellular areas.

The units of arginase administered by Vrat were high, but the preparations were relatively impure; the doses were given in 1 ml. of solution in contrast to the 0.1 ml. employed in our work. In experiments with comparable dosages of arginase of higher purity employed in our work, no such effects were obtained. The influences on body weight and tumor growth are compared in Table 2. Histological examination of the arginase-treated Shimkin mammary carcinoma and the Gardner

[7] Standard deviation, $S^2 = (\Sigma X^2)/(N-1)$; Standard error of the mean, $S_x = S/\sqrt{N}$; Standard error of the difference between means,

$$S^2 = \frac{\Sigma(X_1^2) + \Sigma(X_2^2)}{(N_1 - 1) + (N_2 - 1)} \qquad S_D = \frac{S}{\sqrt{\frac{N_1 N_2}{N_1 + N_2}}}$$

[8] These received five injections of 1 ml. of arginase (80 A.U./ml) at 24-hour intervals.

[9] These received 1 ml. of Co^{++}-activated arginase containing 200 A.U./ml intraperitoneally every other day for 16 days. The controls received equal quantities of 0.1 M phosphate buffer containing 0.125 mg. Co^{++}/ml.

TABLE 2

EFFECT OF ARGINASE TREATMENT ON TUMOR GROWTH IN MICE

EXPER. NO.	TUMOR	INJECTIONS		TOTAL NO.	MORTALITY (per cent)	N†	AV. WEIGHT CHANGE (gm.)	AV. TUMOR WEIGHT (gm.)
		Solution*	Amount					
III	MC-S	Mn^{++}-arginase	125 units/0.1 ml 2×daily	16	30‡	7	4.1±0.6§	2.99±0.31§
		Mn^{++}-buffer	0.1 ml. 2×daily	16	0	9	3.0±0.4	2.84±0.31
		BSA	90 mg/0.1 ml 2× daily	16	20	8	2.9±0.3	2.59±0.24
		Arginine	0.08 mM/0.1 ml 2×daily	16	0	9	3.6±0.2	3.34±0.15
		None			10	9	3.4±0.5	3.02±0.47
VIII	MC in A females	Mn^{++}-arginase	200 units /0.1 ml daily#	12	0	10	1.1±0.6	0.56±0.09
		Mn^{++}-buffer	0.1 ml. daily	12	0	10	1.6±0.2	0.68±0.09
		BSA	1.05 mg/0.1 ml#	12	0	10	1.8±0.2	0.66±0.10
		None			20	8	2.1±0.5	0.56±0.10
	MC in A males	Mn^{++}-arginase	200 units/0.1 ml daily	12	0	10	0.9±0.3	0.58±0.05
		Mn^{++}-buffer	0.1 ml. daily	12	0	10	1.2±0.2	0.49±0.07
XII	MC-S	CO^{++}-Mn^{++}-arginase-1	200 units/0.1 ml daily	12	40‡	6	*−1.5*±1.0	1.12±0.42
		Co^{++}-Mn^{++}-arginase-2	200 units/0.3 ml daily	12	50	5	−0.6±1.2	1.71±0.44
		Co^{++} in 1 per cent Na acetate‖	0.3 ml. daily	12	10	9	1.1±0.4	1.38±0.31
XIII	MC-S	Arginase (Vrat)-1	32 units/0.4 ml 2×daily**	20	0	10	*1.8*±0.3	0.91±0.14
		None			0	10	3.0±0.2	0.87±0.15
		Co^{++}-arginase (Mohamed)	40 units/0.4 ml 2×daily	20	0	4	3.2±0.2	0.99±0.44
		Co^{++} in 1 per Na acetate	0.3 ml. 2×daily	20	0	5	3.1±0.2	0.66±0.25
XIV	MC-S	Arginase (Vrat)-2	15 units/0.5 ml 2×daily**	15	0	15	*1.2*±0.23	*0.45*±0.06
		None			0	15	2.1±0.21	0.72±0.11
V	LS-G	Mn^{++}-arginase	125 units/0.1 ml 2×daily	14	0	4	4.6±1.2	1.44±0.09
		Mn^{++}-buffer	0.1 ml. 2×daily	14	0	4	4.5±0.6	1.42±0.13
IX	LS-G	Mn^{++}-arginase	200 units/0.1 ml daily	8	0	6	*5.3*±0.5	*0.89*±0.02
		Mn^{++}-buffer	0.1 ml. daily	8	0	6	5.4±0.3	0.79±0.04
IV and VI	Asc-E	Mn^{++}-arginase	125 units/0.1 ml 2×daily	14	0	10	8.8±0.8	*8.2* ±0.58††
		Mn^{++}-buffer	0.1 ml. 2×daily	14	0	9	9.3±0.5	6.3 ±0.38††

MC-S = Shimkin mammary carcinoma in C3H mice; MC in A, first transplant of a spontaneous mammary carcinoma in Strain A mice; LS-G = Gardner lymphosarcoma 6C3H-ED in C3H mice; Asc-E = Ehrlich ascites in C3H mice; Mn^{++}-arginase, prepared in presence of Mn^{++} as only activating ion; Co^{++}-arginase, prepared in presence of Co^{++} as only activating ion; arginase (Vrat), received from Permanente Research Laboratories, preparation procedure unknown; BSA-Crystalline bovine serum albumin, Armour.

* Each solute in any experiment was dissolved in the solvent of choice for the arginase solution; i.e., Mn^{++}-maleate buffer or 1 per cent sodium acetate plus Co^{++}.

† N = number of animals used for statistical analysis. Regressions or deaths were not included.

‡ The high mortality rate occasionally observed with MC-S was due to ulceration of tumors which did not seem to be a function of the injections.

§ Standard error of the mean.

Contained 0.068 mg. N per injection.

‖ Dialysate from dialysis of Co^{++}-Mn^{++}-arginase-1 and -2 against 1 per cent sodium acetate plus Co^{++}.

** Subcutaneous injections (as recommended).

†† N = 5; tumor weights from Experiment VI only.

lymphosarcoma showed no consistent or significant differences from the control tumors.[10]

Most of our experiments were with Mn^{++}-activated arginase, which is much more stable than the Co^{++}-activated enzyme. Since the maximum activity of the former is at pH 9.8, it could be argued that this form of arginase is inert at the pH of the body. Evidence that this is not true is offered by the observations of the decrease in blood plasma arginine produced by injection of Mn^{++}-activated arginase. However, in addition, to eliminate this criticism, a small amount of Co^{++}-activated arginase was prepared in our laboratory and tested, with no different results. Samples of arginase prepared in the Permanente laboratory proved ineffective. Retardation of tumor growth, when it occurred, could be correlated with concomitant weight loss of the treated animals and therefore was of no significance.

In the experiments of Neukomm *et al.* (17) with Co^{++}-activated arginase, injections of a total of 700 units of arginase produced no effect on the rate of the tumor growth. Injections with a total dosage of 1640 units produced about a 35 per cent retardation for a period of 2–3 weeks after the injection; this was followed by an increase in the growth rate to those of the control values.

Injection of their Co^{++}-activated arginase produced a subcutaneous hardening, resulting in fistulas in 5–6 days. This was also manifest in our experiments with the arginase preparation obtained from the Permanente laboratory. With this latter solution, the effect may have been the result of injecting the phosphate buffer-Co^{++} solution at pH 6.0. Phosphate at this pH causes a more or less complete precipitation of the cobalt. The calcification reported by Vrat may be related to this.

The experiments reported here on the distribution of injected arginase and its effect on the arginine levels of tumors offer no reason to anticipate that arginase would lead to a highly effective retardation of tumor growth, based on the expectation that it would produce a preferential arginine deficiency in the tumor. The results of injections of arginase on the growth of a variety of tumors substantiate the lack of any significant lasting degree of retardation of tumor growth by this enzyme.

[10] We are indebted to the pathological staff of the Laboratory of Experimental Oncology, University of California, and Dr. M. Shimkim, Director, for performing the histological examinations. Their report in part is as follows: "Comparison of tumors from arginase-treated mice with control tumors revealed no consistent or significant differences. The patterns, areas of necrosis, stroma, appearance of individual cells, and number of mitoses are approximately the same. Specifically, no areas of calcium deposition are encountered in any of the tumors. No histological criteria of effect are seen in the arginase-treated tumors."

SUMMARY

An investigation has been carried out on the relation of arginine and the enzyme arginase to tumor growth. The more significant results obtained are as follows:

1. Contrary to previously published results, no statistically significant differences were found in the arginase activities of blood plasma, muscle, or liver between control and tumor-bearing mice.

2. Intraperitoneal injection of a large number of units of arginase induced a considerable but transient drop in the plasma arginine level and no significant changes in the arginine concentration of tumor or liver tissue. The arginase content of the blood plasma was greatly increased after the injection for a period of up to 24 hours, but tumor tissue showed only a comparatively small increase which lasted only for a few hours.

3. No persistent carcinostatic effects were observed on a spectrum of tumors by the continued injection of daily doses of a large number of arginase units.

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ADDENDUM

The following article appeared after this manuscript went to press:

BACH, S. J., and SIMON-REUSS, I. Arginase, An Antimitotic Agent in Tissue Culture. Biochim. et Biophys. Acta, **11**: 396, 1953.

Metabolism of the DBA Mouse Ascites Thymoma*

HILTON B. LEVY,† HAROLD M. DAVIDSON,‡ ROBERT W. REINHART,† AND ARTHUR L. SCHADE†

(Overly Biochemical Research Foundation, New York, N.Y.)

INTRODUCTION

In preparation for an investigation of protein synthesis by the cells of a mouse ascites thymoma and the energetics related to such synthesis, we wished to establish the metabolic characteristics of this tumor as a basis for the projected work. The tumor we employed was a transplantable lymphoma which arose originally as a tumor of the thymus in a DBA 212 mouse at the National Cancer Institute.[1] Previous study of this tumor has been primarily concerned with the cultural growth characteristics of free tumor cells in the peritoneal fluid (4) and with their average nucleic acid content per cell as correlated with their capacity for growth (3).

In conjunction with the establishment of the metabolic quotients of the lymphoma (Q_{O_2}, $Q^{O_2}_{CO_2}$, $Q^{N_2}_{CO_2}$, $Q^{O_2}_{lactic}$, $Q^{N_2}_{lactic}$, and R.Q.), we investigated the relative amounts of pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) of the tumor cells at various age levels of the tumor. The most convenient way to have tumors of similar "age" is to use tumor cell suspensions after the same number of days' incubation of comparable inocula in the peritoneal cavities of the host mice maintained under standardized conditions. In our experimental work, groups of about 25 animals were inoculated uniformly with an ascites thymoma cell suspension, and at certain times covering the period 4–14 days after inoculation, four to six animals were sacrificed, the ascites tumors pooled, and studied. While our experience with many generations of this tumor has indicated that a given chronological age does not necessarily assure a definite "biological age," we have observed in appropriate experiments a consistent variation in some metabolic activities of the tumor with its increasing chronological age. That such variation does occur is of considerable interest in itself and of importance for contemplated biochemical and physiological studies with this tumor.

Tiedemann (14) determined several metabolic quotients of washed Ehrlich ascites tumor cells in salt solutions. He found that the cells had high glycolytic capacities and that glucose had an inhibitory effect on the respiration. Warburg and Hiepler (16) found that the metabolic quotients of the Ehrlich tumor when measured in ascitic fluid were even higher than those obtained by Tiedemann in salt solution. Kun, Talalay, and Williams-Ashman (7) have reported on the metabolism of the Ehrlich ascites tumor. They used washed tumor cells of unspecified age, freed of the ascitic fluid, in their determinations. We have employed both washed and unwashed cells of the DBA thymoma for comparative purposes. Because we believe the metabolism of the unwashed cells in ascitic fluid represents more nearly that of cells present in the peritoneal cavity of the host, most of our work has been done with such cells. A preliminary report of some of this work has been presented (8).

MATERIALS AND METHODS

The DBA thymoma was maintained by repeated transfers on an ascites tumor in the DBA/2 male mouse. All attempts to carry the tumor in the DBA/1 failed, while in line 2 all transfers were successful. Ordinarily, the inoculum was 0.2 ml. of an ascitic cell suspension obtained from a 7–10-day-old tumor. The number of cells thus inoculated into the peritoneal cavity of the mouse varied between 35 and 50 million. The mice were kept at 27° C. ± 2° C. and were fed a regular diet of laboratory chow (Derwood Mills) and water ad libitum.

For the manometric tests, the ascites cell suspension was used directly in ascitic fluid, in Krebs-

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† Present address: National Microbiological Institute, National Institutes of Health, Bethesda, Maryland.

‡ Present address: Boston Dispensary, 25 Bennet Street, Boston 11, Mass.

[1] Through the generosity of Dr. T. Hauschka of the Institute for Cancer Research, Philadelphia, we were given a transplant of this tumor first obtained by that Institute from Dr. Dalton of the National Cancer Institute.

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Ringer phosphate (K-R.p.), or in Krebs-Ringer bicarbonate (K-R.c.) media (15). To each milliliter of the cell suspension was added 30 μg. of heparin.[2] As short a time interval as possible was allowed to elapse between the time of removal from the peritoneum of the cell suspension and the first manometric reading of its metabolic activity. This time interval seldom exceeded 30 minutes. When washed cells were used, they were prepared by diluting the ascites cell suspension in about 5 times its volume of 0.9 per cent NaCl solution (saline), centrifuging at 2°–4° C. in a Servall SS-1 centrifuge at 55 v. (approx. 2,200 × *g*) for 5 minutes, and resuspending the cells in a volume of saline roughly equal to 6 times the original volume of cell suspension. After recentrifuging under similar conditions, the loosely packed cells were diluted to a given concentration in the desired medium and used immediately.

The dry weight of cells in a tumor suspension was obtained by washing a known volume of cells in the manner described above, making the cells up to a known volume with saline, drying an aliquot of the washed cell suspension to constant weight at 105° C., and correcting for the weight of NaCl in the suspending liquid. For the dry weight determination, aliquots of cells were taken off prior to, rather than after, incubation.

For the determination of cell number, a measured volume of the ascites tumor cell suspension was diluted in a suitable volume of physiological saline and lightly stained with gentian violet. All counts were then made on the diluted suspension in a hemocytometer.

The volume of cells/ml of cell suspension was obtained by centrifuging 1 ml. of the suspension in a centrifuge tube with a graduated capillary tip, in an International No. 2 centrifuge at 1,600 × *g* for 15 minutes. The cell volume/ml divided by the number of cells/ml gave the average volume per cell.

The turbidity of a cell suspension was determined as follows: 0.1 ml. of cell suspension was diluted with 15 ml. of saline and read in a Pfaltz and Bauer fluorophotometer which had been set to give a galvanometer reading of 50.0 with a Pfaltz and Bauer vitamin B_1 glass standard. The determinations were made using the regular Pfaltz and Bauer rectangular cell and were corrected for the reading made with plain saline in the same cell.

Q_{O_2} determinations were usually made in the conventional one- or two-armed Warburg respirometer vessels with KOH in the center well and K-R.p. buffer (pH 7.4) or ascitic plasma plus glucose (which was decarbonated and adjusted to pH 7.4 by careful addition of H_3PO_4) as diluents of the tumor cell suspension in the main compartment. To assess the effect on the Q_{O_2} of a CO_2 pressure more nearly representative of the conditions obtaining in the peritoneum itself than those established by the KOH in the experimental vessel, we determined the Q_{O_2} with Pardee's solution (11) in the center well. The CO_2 in the gas phase was maintained at approximately 2 per cent. The Pardee solution was stabilized against autoxidation by addition of thiourea, as recommended by Krebs (6). The control thermobarometer was set up with Pardee solution in its center well to correct for the residual autoxidation which did take place.

The $Q^{N_2}_{CO_2}$ values were ordinarily determined by Warburg's direct method in which the gas phase was 95 per cent N_2 and 5 per cent CO_2 and the suspending fluid was either the ascitic plasma or bicarbonate buffer (K-R.c.) plus glucose. When ascitic plasma was used, correction was made for CO_2 retention, usually by multiplying the observed manometric change in millimeters by four-thirds.

The $Q^{O_2}_{lactic}$ and $Q^{N_2}_{lactic}$ values were determined by means of the Barker and Summerson lactic acid method as outlined in Umbreit (15). Incubation of the tumor cell suspension with glucose was carried out in undiluted ascitic plasma or in ascitic plasma diluted with 4 times its volume of Krebs-Ringer carbonate. The gas phase was 5 per cent CO_2 plus 95 per cent O_2 or 95 per cent N_2, and the duration of incubation was 40–60 minutes. These quotients were also determined for washed cells in K-R.c. plus glucose.

R.Q. and $Q^{O_2}_{CO_2}$ values for the thymoma suspension in ascitic fluid diluted 1:1 with K-R.c. plus glucose were obtained through use of Summerson differential manometers (13). Concurrent Q_{O_2} determinations were also made.

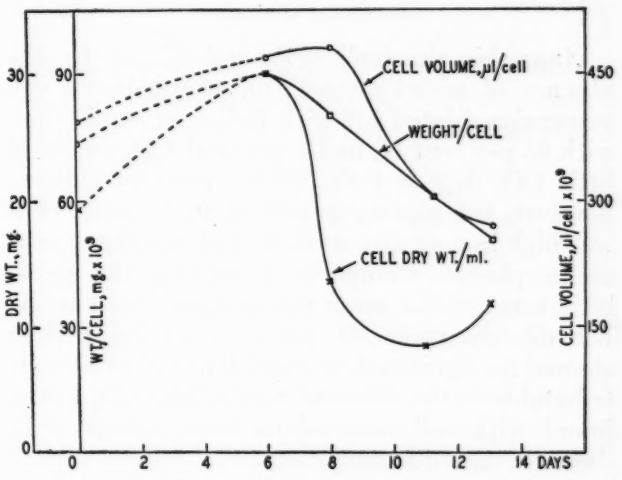

CHART 1.—Physical characteristics of ascites thymoma as affected by "age" of tumor. The inoculum was a mixture of cells 9–13 days old. The dry weight/ml data represent the dry weight of cells/ml of ascitic cell suspension.

The nucleic acids were extracted from the cells with hot trichloroacetic acid, after washing with saline and thorough extraction with cold trichloroacetic acid to remove acid-soluble nucleotides (12). Pentosenucleic acid (PNA) was determined colorimetrically through the use of von Euler's phloroglucinol reaction (2) and desoxypentosenucleic acid (DNA) by Dische's diphenylamine procedure (1). The total nucleic acids so obtained agreed well with the total nucleic acid concentration obtained through measurement of the optical absorption at 260 mμ.

RESULTS

PHYSICAL AND CULTURAL CHARACTERISTICS OF THE CELL SUSPENSION

Examination of the course of the development of the ascites tumor with respect to cell weight, cell number, and cell volume, all per milliliter of suspension, gave the results expressed in Chart 1.

It will be seen that the cell dry weight/ml of suspension reached a maximum about 6 days after inoculation, and then declined. The number of cells/ml simultaneously reached a maximum and

[2] Heparin, free of preservative, was kindly supplied by Lederle Laboratories, through the courtesy of Dr. Stirn.

then declined, but the changes were not so great as the changes in dry weight. As a consequence, the weight per cell declined with time after reaching a maximum. The volume per cell changed in a manner similar to the weight per cell. The cell suspension was either free of blood or slightly hemorrhagic.

Within about 16 days after inoculation, most of the animals were dead, although some lived as long as 3 weeks. It is of interest that intraperitoneal inoculation of a small amount of blood from the heart or tail vein of an infected animal gave rise to a regular ascites thymoma within about 2 weeks.

Metabolic Quotients

Anaerobic glycolysis ($Q^{N_2}_{CO_2}$ *and* $Q^{N_2}_{lactic}$).—In the absence of added glucose, an ascitic tumor cell suspension diluted 1:5 with K-R.c. in equilibrium with 95 per cent N_2 and 5 per cent CO_2 produced little CO_2 ($Q^{N_2}_{CO_2}$ = 1–2). With 1 per cent glucose, however, the glycolytic rate of such tumor cells was high in a similar medium and in 100 per cent ascitic plasma. Comparisons between the glycolytic rates of the same tumor suspensions in K-R.c.-diluted and 100 per cent ascitic plasma showed no significant or consistent differences attributable to the different media. The $Q^{N_2}_{CO_2}$ values found with cell suspensions from tumors 4–14 days old varied from 33 to 83.

In any given series in which tumors were studied over a period of days, the glycolytic Q values increased with increasing age of tumor. For example, in three different series the following values were found: 39 and 73 for a 4- and 7-day-old tumor, respectively; 33 and 56 for a 9- and 13-day-old tumor, respectively; and 49 and 81 for a 6- and 11-day-old tumor, respectively. It is clear that the absolute Q values for any given age of tumor from different series vary considerably. Chemical lactic acid determinations gave $Q^{N_2}_{lactic}$ values which were in accord with the manometric $Q^{N_2}_{CO_2}$ values.

A few experiments using washed cells suspended in K-R.c. gave $Q^{N_2}_{CO_2}$ and $Q^{N_2}_{lactic}$ values similar to those in the unwashed cells in K-R.c. and ascitic plasma.

Aerobic glycolysis ($Q^{O_2}_{CO_2}$ *and* $Q^{O_2}_{lactic}$).—Aerobic glycolysis of 1 per cent glucose by the ascites thymoma was determined in ascites plasma and in ascitic plasma diluted 1:5 with K-R.c. The gas phase was 95 per cent O_2 and 5 per cent CO_2. The $Q^{O_2}_{lactic}$ values observed varied from 8 to 28 and were found to increase with increasing age of the tumor, as did the anaerobic Q_{lactic} values. A typical series gave figures of 8.4, 16, 21.3, and 27.9 for the $Q^{O_2}_{lactic}$ of ascitic cell suspensions 6, 8, 11, and 13 days after inoculation of the host mice. In the experiments where $Q^{O_2}_{CO_2}$ was determined manometrically by Summerson differential manometers, there was correspondence with the $Q^{O_2}_{lactic}$ so that all the acid produced under aerobic conditions by these thymoma cells was lactic acid. The aerobic glycolysis of any given tumor cell suspension was only 30–50 per cent of the anaerobic glycolysis of the same cells.

Washed thymoma cells in K-R.c. plus 1 per cent glucose showed aerobic glycolytic activity similar to those of unwashed cells and of a value 30 per cent of their anaerobic glycolytic activity.

Respiration (Q_{O_2}).—The Q_{O_2} of unwashed tumor cells suspended in ascitic plasma and in ascitic plasma diluted fivefold with K-R.c. or K-R.p., in the presence or absence of glucose, was found to vary between 10 and 16 in air and in oxygen. The average value was 13. No consistent difference was observed in the Q_{O_2} values determined in the presence of physiological concentrations of CO_2 or in its virtual absence. Furthermore, in contrast to the anaerobic and aerobic glycolysis of the ascites thymoma, the respiration did not increase or change consistently with increasing age of the tumor.

As a consequence of the fact that the absolute values of $Q^{N_2}_{CO_2}$ and $Q^{O_2}_{CO_2}$ rose as the age of tumor increased, with the ratio between them remaining essentially constant, the absolute Pasteur effect increased. Since the Q_{O_2} did not rise proportionately, the Meyerhof quotient $(Q^{N_2}_{lactic} - Q^{O_2}_{lactic}/Q_{O_2})$ also increased with advancing age of tumor. For example, a 6-day-old tumor showed a Meyerhof quotient of 27.7 − 8.4/12.1 or 1.6, while the quotient of the 11-day-old tumor was 64.3 − 21.3/13.2 or 3.26.

Kun *et al.* (7), working with washed cells of the Ehrlich ascites carcinoma, found that the rate of oxygen uptake by those cells in 100 per cent oxygen was invariably depressed 40–60 per cent by glucose over a concentration range of 0.4–1 per cent. Using washed cells of the ascites thymoma suspended in K-R.p. in an atmosphere of oxygen, we tested the effect of 0.25 per cent, 0.5 per cent, 1.0 per cent, and 2.0 per cent glucose on the Q_{O_2}. No inhibitory effect of glucose on the oxygen uptake by these tumor cells was observed.

The respiratory quotients (R.Q.) of thymoma cell suspensions were determined in ascitic plasma diluted 1:1 with K-R.c. in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 with Summerson differential manometers. The results show that the R.Q.'s, of young tumors (4–6 days) range from 0.82 to 0.85, while those of old tumors (10–14 days) rose to values varying from 0.88 to 0.93.

NUCLEIC ACID

Since the preceding data have indicated that there is a variation with age of some metabolic activities of the ascites thymoma, it was of interest to examine the tumor cells for a possible corresponding variation in their nucleic acid content. To this end, the concentrations of PNA and DNA in these cells as a function of the time after inoculation were determined. Chart 2 illustrates the variation of PNA concentration of the ascites cells with time after inoculation of the tumor, the PNA content being expressed as μg PNA/cell and as percentage of the dry weight of the cells.

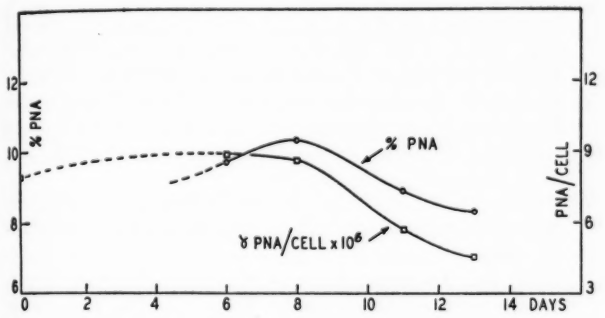

CHART 2.—PNA content of ascites thymoma as affected by "age" of tumor. Inoculum as in Chart 1.

It will be seen that the percentage PNA reached a maximum at about the same time as, or just slightly later than, the time of maximum cell population[3] (6th–7th day, as shown in Chart 1), and then declined by about 25 per cent. As a consequence of the decline in the percentage of PNA and the concurrent decrease in cell size and weight (cf. Chart 1), the PNA per cell dropped even more markedly.

The percentage of DNA also changed with the age of the tumor. In one case the percentage of DNA rose from an initial value of 10 per cent to 12 per cent at the 6th day and then declined to about 10 per cent after 13 days. During a comparable period of time, the tumor cells used to obtain the data of Chart 3 showed a steady rise from 11 to 15 per cent. It is likely that longer observation would have revealed a decline in this case also. However, in all cases the cyclic increase and decrease in cell size resulted in a corresponding rise and fall in DNA per cell. In the case illustrated in Chart 3 the maximum was 11.3×10^{-6} μg/cell, at 6 days, while at 13 days the value had fallen to 8.8×10^{-6} μg/cell.

[3] For the purpose of enabling direct comparison, the series of experiments chosen for presentation of the nucleic acid data is the same series that was used for the data of Chart 1.

DISCUSSION

The regular variation in DNA per cell found here is not necessarily inconsistent with the rather widely held view that the DNA content per nucleus is constant for a given species, because that hypothesis was developed primarily for resting tissues, while we are here dealing with a growing tissue. If the period of time required by the cell in preparation for and completion of mitosis—during which period the DNA concentration increases —were of suitable length (2–4 hours), then a changing percentage of cells preparing to divide or in division over the period of observed tumor growth could account for our cyclic changes in average DNA per cell. On this basis, of course, one would expect maximum mitotic activity when the DNA per cell is maximal, i.e., 6–8 days after inoculation (Chart 3). That the cells are undergoing maximum protein synthesis at this time is suggested by the fact that the PNA per cell is highest then (Chart 2).

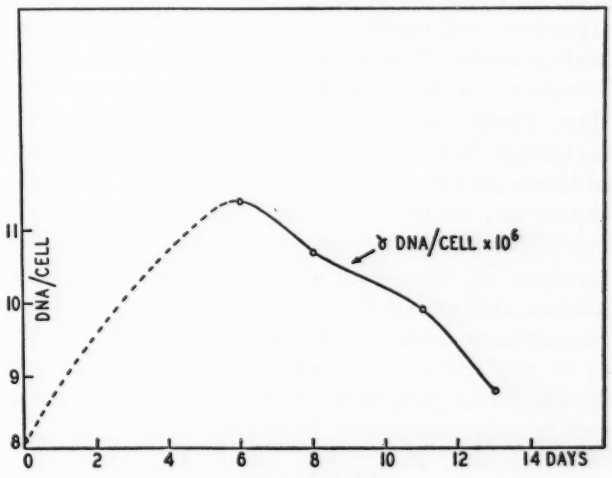

CHART 3.—DNA content of ascites thymoma as affected by "age" of tumor. Inoculum as in Chart 1.

Klein (5) determined the nucleic acid concentration of a group of ascites tumors without particular attention to the length of time after inoculation. He found that his ascites thymoma cells did not differ in size among themselves nor from normal lymphocytes. He also found no deviation in DNA per cell from the value usually given for normal mouse somatic cells. Our lymphoma ascites cells, on the other hand, varied in size during their growth cycle (Chart 1), and during at least part of the cycle had higher than normal DNA per cell. Our lymphoma cells, therefore, are more like Klein's nonlymphoma ascites tumors, in which he also found increased cell size and high DNA per cell.

Since the changes in dry weight of cell substance/ml of cell suspension (Chart 1) are largely attributable to the host's production of ascitic fluid, one cannot assume that the time when one finds the maximum cell substance/ml is the time of maximum cell reproductive rate. The difficulty of removing all the cells from the peritoneal cavity makes hard the determination of total cell weight as a function of time after inoculation. However, the combination of circumstantial evidence presented in Charts 1, 2, and 3 makes it plausible that the ascites tumor cell suspension undergoes a period of rapid growth followed by a decline in growth rate, not unlike that found in micro-organisms. (For a comparison of nucleic acid concentration and cell growth rate in micro-organisms see [9].)

A number of differences between the DBA thymoma and the Ehrlich carcinoma are worth noting. Tiedemann (14) and Kun *et al.* (7) reported that glucose inhibits the respiration of washed Ehrlich ascites carcinoma cells. We have repeated and confirmed this with both washed and unwashed Ehrlich carcinoma cells. We found, however, no such effect with the thymoma. Further, Tiedemann found, with the Ehrlich tumor, no change in the glycolytic activities as a function of the number of days after inoculation. With the thymoma, we observed very definite increases in glycolysis as the tumor grew older. Marked differences in the aldolase activity of the blood plasma and ascitic fluids of animals bearing these tumors have been reported elsewhere (10). Whether or not these differences are reflections of any fundamental metabolic differences between these two tumors remains to be determined.

SUMMARY

Some metabolic characteristics of an ascites thymoma in DBA mice, as a function of the number of days after inoculation of the tumor into the host, have been determined. The weight/cell, volume/cell, and cell dry weight/ml of cell suspension rose to a maximum 6–8 days after inoculation and then declined. The amount of PNA/cell and of DNA/cell also showed a cyclic rise and fall. The R.Q., $Q^{O_2}_{CO_2}$, and $Q^{N_2}_{CO_2}$ increased with age, the latter reaching values as high as 83. The Q_o remained essentially constant during the course of the growth of the tumor. It is suggested that the ascites tumor undergoes a period of rapid proliferation, followed by a decline in growth rate.

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Studies on the Ehrlich Ascites Tumor

II. Oxidation of Hexose Phosphates*

H. G. WILLIAMS-ASHMAN†

(Ben May Laboratory for Cancer Research, University of Chicago, Chicago 37, Ill.)

A previous report from this laboratory (8) demonstrated that washed Ehrlich ascites tumor cells showed high rates of anaerobic and aerobic glycolysis which were completely dependent upon the addition of glycolyzable hexoses. The respiration of this tissue, however, was considerable and persistent in the absence of added sugar. The initial rate of oxygen consumption was either unaffected or actually depressed by the addition of glucose and fructose, the exact effect of these sugars depending upon the experimental conditions employed. Warburg and Hiepler (15) have since reported similar glycolytic and respiratory activities for unwashed preparations of this tumor.

During the course of studies on the influence of hexoses on various synthetic reactions carried out by ascites tumor cells under anaerobic and aerobic conditions, it became of interest to examine the possible metabolic pathways for the degradation of added glucose. In this connection the direct oxidation of hexose phosphates, as opposed to their conversion to pyruvate via the Embden-Meyerhof-Parnas glycolytic sequence, was investigated. Since the discovery of the oxidative decarboxylation of G-6-P[1] in erythrocytes by Warburg and Christian (13), the direct oxidation of G-6-P and 6-P-G has been shown to occur in a number of animal tissues (3, 6). Relatively little is known concerning the participation of this "hexose monophosphate shunt" mechanism in malignant tissue. Dickens and Glock (3) have reported rapid rates of oxidation of G-6-P and 6-P-G by extracts of a liver tumor; the activity of the Rous sarcoma was rather feeble.

The experiments reported in this paper demonstrate that the Ehrlich ascites tumor contains active G-6-P and 6-P-G dehydrogenases. The activity of these catalytic systems is compared to that of certain glycolytic and respiratory enzymes in this tumor, which is also shown to contain a powerful glutathione reductase.

METHODS

Materials.—G-6-P dehydrogenase and alcohol dehydrogenase were prepared from bakers' yeast according to Kornberg (7) and Racker (10), respectively. The G-6-P dehydrogenase was free of 6-P-G dehydrogenase activity.

DPN (purity 0.58–0.64) and TPN (purity 0.48–0.53) were obtained from Schwartz Laboratories. The DPN was further purified by chromatography on Dowex anion exchange resins according to an unpublished procedure of A. Kornberg and B. L. Horecker. The purity of the specimens of DPN used in the experiments described below ranged from 0.80 to 0.84. The TPN was free of DPN and vice versa. DPNH and TPNH were prepared from solutions of the corresponding oxidized nucleotides by reduction with hydrosulfite according to Gutcho and Stewart (4).

G-6-P and R-5-P were obtained as the barium salts from Schwartz Laboratories. 6-P-G was synthesized according to Seegmiller and Horecker (12), the product containing less than 1 per cent G-6-P. 2-P-G was synthesized by the method of W. Kiessling as described by Warburg and Christian (14). GSSG was obtained from Schwartz Laboratories. DL-isocitric acid was generously donated by Dr. T. W. Rall. All compounds were dissolved in deionized water as the corresponding sodium salts.

Estimations.—DPN was determined with alcohol dehydrogenase according to Racker (10). TPN was determined with the aid of G-6-P dehydrogenase (7). All spectrophotometric measurements were made with a Beckman DU quartz spectrophotometer with quartz cells of 1 cm. light path.

Preparation of acetone powders.—The ascitic fluid was removed from the intraperitoneal cavities of the mice with a syringe and immediately centrifuged at 0°. The ascitic plasma was removed and the cells washed 5 times with 0.9 per cent NaCl. Fluids containing more than 2 per cent erythrocytes were discarded, and great care was taken to remove any erythrocytes which sedimented during the centrifugations. The final washed pellet of cells was quickly added to at least ten volumes of acetone (−25°) and comminuted in a Waring Blendor in the cold room for 1 minute. The insoluble material was collected on a Buchner funnel and washed 5 times without allowing the filter cake to crack. The powder was dried *in vacuo* over $CaCl_2$ and stored in a desiccator at −25°.

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† Scholar in Cancer Research of the American Cancer Society.

[1] The following abbreviations are employed: DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; DPNH = reduced DPN; TPNH = reduced TPN; G-6-P = glucose-6-phosphate; 6-P-G = 6-phosphogluconate; 2-P-G = 2-phosphoglycerate; R-5-P = ribose-5-phosphate; GSH = reduced glutathione; GSSG = oxidized glutathione; TRIS = tris(hydroxymethyl)amino-methane.

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Extraction of the acetone powders.—The powder was weighed out in a centrifuge tube and cooled in an ice bath. Cooled extraction medium was slowly added, the mixture being rubbed into a uniform paste with a glass rod. After all the fluid had been added (10–20 volumes), the slurry was allowed to stand for 1 hour at 0° with intermittent stirring. In some experiments the mixture was also homogenized for 1 minute in an all-glass homogenizer immersed in an ice bath. The mixture was centrifuged at 4,000 × *g* and the supernatant fluid poured through five layers of gauze. It was then centrifuged again and any precipitate discarded.

Other methods are described elsewhere (8).

RESULTS

Oxidation of G-6-P and 6-P-G by acetone powder extracts.—Extraction of the acetone powders with either 0.005 M $NaHCO_3$ or 0.1 M glycylglycine pH 7.5 gave clear extracts which readily reduced

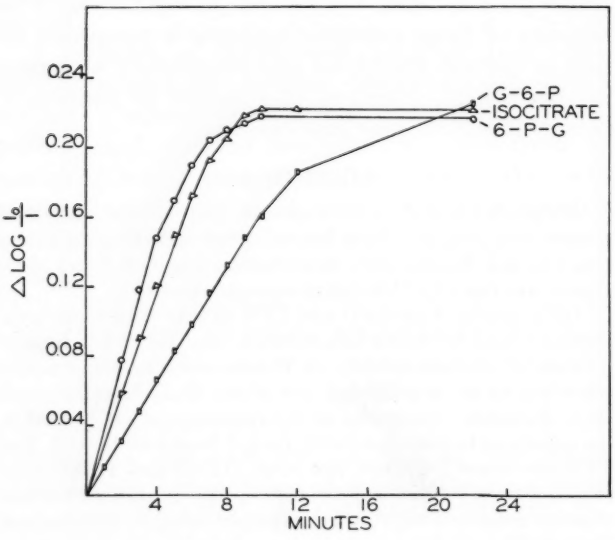

CHART 1.—Oxidation of glucose-6-phosphate, 6-phosphogluconate, and isocitrate by Ehrlich ascites tumor extract. Test system as in Table 2 except that 0.11 μM TPN added to all cuvettes.

TPN in the presence of G-6-P and 6-P-G. The reactions were followed by measuring the increase in light absorption at 340 mμ owing to the formation of TPNH. Unless otherwise stated, all estimations were performed within 3 hours after extraction of the powders, the extracts being kept at 0° prior to addition to the reaction mixture. The blank cells always contained all the components of the reaction mixture except the substrate in order to compensate for any endogenous TPN reduction. This proved to be negligible, however, over the 5–10-minute periods at 24°–26° C. used for the determination of hexose phosphate oxidation. Chart 1 shows the change in light absorption at 340 mμ due to the reduction of TPN by G-6-P, 6-P-G, and isocitrate. This experiment demonstrates that more than 95 per cent of the added TPN was reduced when the reaction had gone to completion and that after this time the light absorption at 340 mμ remained constant, suggesting that such extracts do not contain enzymes capable of rupturing the nicotinamide-ribose bond of either TPN or TPNH. Other experiments with limiting amounts of substrate showed that after the reduction of TPN was complete, no further change in light absorption occurred, suggesting that the extracts were incapable of oxidizing TPNH. The light absorption at 400 mμ was unchanged in these experiments.

In attempts to assess the possible contribution of hexose phosphate oxidation to the over-all respiration of the intact ascites tumor cells, the activity of the G-6-P and 6-P-G dehydrogenases was compared to that of the lactic and isocitric dehydrogenases present in the extract. These measurements were made under conditions where the change in optical density at 340 mμ was proportional to the volume of extract added and in the presence of optimal concentrations of substrates and co-factors. Typical results obtained with two different samples of powder are shown in Table 1.

TABLE 1

RELATIVE DEHYDROGENASE ACTIVITIES OF EHRLICH ASCITES TUMOR

DEHYDROGENASE ACTIVITY	UNITS/MG ACETONE POWDER*	
	Sample I	Sample II
Lactic	241	
Isocitric	17.6	16.8
Glucose-6-phosphate	9.7	9.0
6-Phosphogluconic		18.4

* One unit is equivalent to a change in optical density of 0.001 per minute at 25°. All reactions carried out in final volume of 3.0 cc. Lactic dehydrogenase system contained 100 μM phosphate buffer pH 7.4; 0.3 μM DPNH and 5 μM sodium pyruvate; reaction followed by measuring decrease in light absorption at 340 mμ; rate corrected for slight oxidation of DPNH in the absence of pyruvate. Test systems for other dehydrogenase systems as in Table 2 except that 100 μM glycylglycine pH 7.4 was used as buffer in place of TRIS.

It can be seen that the 6-P-G dehydrogenase activity was of the same order of magnitude as the isocitric dehydrogenase activity, whereas the G-6-P dehydrogenase was approximately one-half as active as these enzymes. The lactic dehydrogenase activity was about 14 times that of the isocitric dehydrogenase.

It is not possible to make a strict comparison of these activities in fresh tissue acetone powder extracts with those observed for lactic dehydrogenase by Wenner, Spirtes, and Weinhouse (16) and for 6-P-G dehydrogenase by Horecker and Smyrniotis (6), since these investigators employed reaction mixtures of different total volume and composition from those employed in the present studies. However, the values reported in Table 1

are in rough accord with the values observed by these workers for a number of animal tissues. It may be remarked that, although the comparison of the rates of hexose phosphate oxidation with those of isocitrate and the lactic dehydrogenase reaction give accurate values for the *relative* activity of these enzyme systems, expression of the individual enzyme activities in terms of unit dry weight of acetone powders is fraught with uncertainties. With a given sample of powder the rates observed varied considerably from one extraction to another, especially when the procedure consisted simply of trituration with the medium at 0°. Homogenization of the powder with the medium appeared to give somewhat higher values for the activity of the enzymes oxidizing hexose phosphates. Grinding washed ascites tumor cells with alumina powder (9) and small amounts of 0.1 M glycylglycine pH 7.4, followed by centrifugation at 14,000 × *g* for 20 minutes at 0°, gave clear extracts which catalyzed the reduction of TPN by G-6-P and 6-P-G. However, the considerable endogenous reduction of TPN by these extracts and their proclivity to reoxidize TPNH made them unsuitable for the comparative study of hexose phosphate oxidation. Nevertheless, the fact that the acetone powder extracts oxidized G-6-P and 6-P-G at comparable rates to isocitrate suggests that the direct oxidation of hexose phosphates may well play an important role in the metabolism of the intact ascites tumor cell.

In the presence of glycylglycine buffer, inorganic phosphate (0.006 M) did not affect the oxidation of G-6-P. Mg^{++} ions were always added to the test system when the rates of hexose phosphate oxidation were compared to that of isocitrate. High concentrations of Mg^{++} ions (0.01 M) were found to give troublesome precipitations with some of the extracts, but at the concentrations employed in these experiments no turbidity was present. The addition of KCl (0.01 M) to reaction mixtures to which no K^+ ions had been added did not affect the reduction of TPN by G-6-P or 6-P-G.

The effect of aging bicarbonate extracts at 2° is shown in Table 2. The G-6-P dehydrogenase was fairly unstable under these conditions, whereas the ability of the extracts to oxidize 6-P-G remained unimpaired for a considerable time. The dry acetone powders retained both G-6-P and 6-P-G dehydrogenase activities for many months if stored *in vacuo* at −25°.

At 25° in the presence of either glycylglycine or TRIS buffers of pH 7.4, no reduction of TPN or DPN was observed with glucose (0.08 M), and the addition of R-5-P (0.003 M) failed to reduce TPN.

Coenzyme specificity.—Extracts containing powerful G-6-P and 6-P-G dehydrogenase activity failed to reduce DPN (0.4–0.8 μM per cuvette) in the presence of either G-6-P or 6-P-G. Pyridine nucleotide transhydrogenase (2) appeared to be absent from these extracts. An experiment designed to demonstrate the presence of this enzyme is shown in Chart 2. In the presence of limiting concentrations of TPN and excess G-6-P, the reduction of TPN was followed at 340 mμ until the reaction had reached completion. DPN was then added, and no change in light absorption at 340 mμ occurred, showing that DPN was not reduced by G-6-P and demonstrating the absence of pyridine nucleotide transhydrogenase. The decrease in light absorption following the further addition of GSSG is attributable to the glutathione reductase in the extract.

TABLE 2

STABILITY OF TPN LINKED DEHYDROGENASES

Duration of storage at 2° (hrs.)	Relative rate of TPN reduction by G-6-P	6-P-G	Isocitrate
0	100	100	100
18	65	103	85
66	12	74	37
138	6	67	22

Reactions were carried out in a final volume of 3 cc. and initiated by the addition of TPN (0.25 μM). Each cuvette contained 100 μM TRIS buffer of pH 7.4; 10 μM $MgCl_2$ and 0.1 cc. of extract (210 mg. powder extracted with 11 cc. of 0.005 M $NaHCO_3$). The following amounts of substrate were added: G-6-P, 21 μM; 6-P-G, 5 μM; isocitrate, 14 μM. Rate of reaction followed by change in light absorption at 340 mμ from 1- to 4-minute period after the addition of TPN (present in blank cells with extract). Rates with fresh extract with all three substrates taken as 100; rates with stored extracts expressed as percentage of initial rate. Ratio of activities of G-6-P:6-P-G:isocitrate with fresh extract = 1:2.06:1.88. Temperature, 25° C.

Coupling of G-6-P and 6-P-G dehydrogenases with glutathione reductase.—Reduction of GSSG to GSH by TPNH was observed in the presence of these acetone powder extracts. Chart 3 demonstrates that the absorption at 340 mμ due to the reduction of TPN by limiting amounts of 6-P-G was abolished by the further addition of GSSG. Experiments with limiting concentrations of G-6-P gave identical results. The rate of oxidation of TPNH (generated *in situ* by limiting concentrations of G-6-P) was roughly proportional to the amount of extract added, suggesting that nonenzymatic oxidation of TPNH by GSSG (1) could not account for these observations. The glutathione reductase activity of these extracts appeared to be completely TPNH specific. Chart 4 shows that DPNH was not oxidized by GSSG, the further addition of pyruvate giving rise to a rapid oxidation of DPNH by the lactic dehydrogenase present in the extract.

Oxidation of hexose phosphates by ascitic plasma.—Ascitic plasma obtained from ascitic fluids virtually free of erythrocytes was found to oxidize both G-6-P and 6-P-G, no endogenous reduction of TPN being observed. The activity of these enzymes was compared to that of the enolase present, the latter enzyme being determined by the increase in light absorption at 240 mμ due to the 2-phosphopyruvate formed from 2-P-G. No enolase activity was observed in the absence of added Mg^{++} ions. The amounts of substrates transformed in these reactions were calculated assuming a millimolar extinction coefficient ($\log_{10} \frac{I_0}{I}$) of 6.22 for TPNH (5) and of 1.63 for 2-phosphopyruvate at 240 mμ (calculated from the data of Warburg and Christian [14] for the absorption of 2-phosphopyruvate at the pH and Mg^{++} ion concentration used for the determination of enolase activity). Table 3 shows that the ability of the ascitic plasma to oxidize G-6-P and 6-P-G was relatively low, the enolase activity being magnitudes higher.

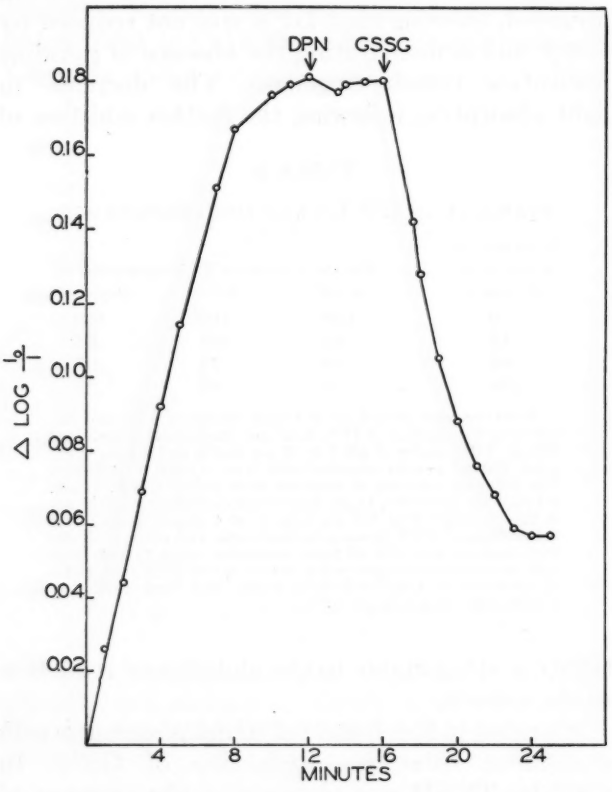

CHART 2.—Absence of pyridine nucleotide transhydrogenase. Cells originally contained 0.087 μM TPN, 0.1 cc. extract (in 0.1 M glycylglycine pH 7.4), 100 μM TRIS pH 7.4 and 10 μM G-6-P in 2.9 cc. At first arrow, 0.1 cc. DPN (0.4 μM) added. At second arrow, 0.1 cc. GSSG (5 μM) added. Change in optical density measured at 340 mμ. Temperature, 25° C.

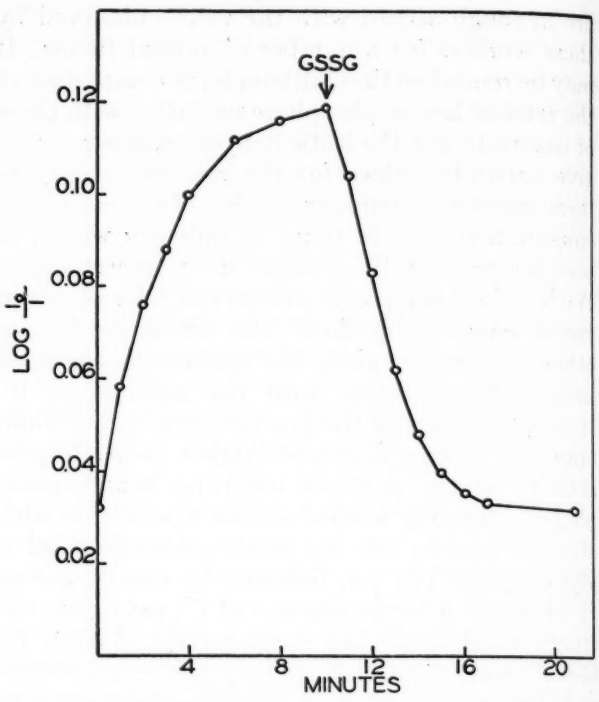

CHART 3.—Glutathione reductase activity of ascites tumor extract. Cells originally contained 100 μM TRIS pH 7.4, 0.31 μM TPN and 0.042 μM 6-P-G in 2.9 cc. Reaction initiated by the addition of 0.1 cc. extract (powder extracted with 0.1 M glycylglycine pH 7.4). At arrow, 0.1 cc. GSSG (5 μM) added. Blank cells contained all components of reaction mixture except 6-P-G. Temperature, 26° C. Wave-length, 340 mμ.

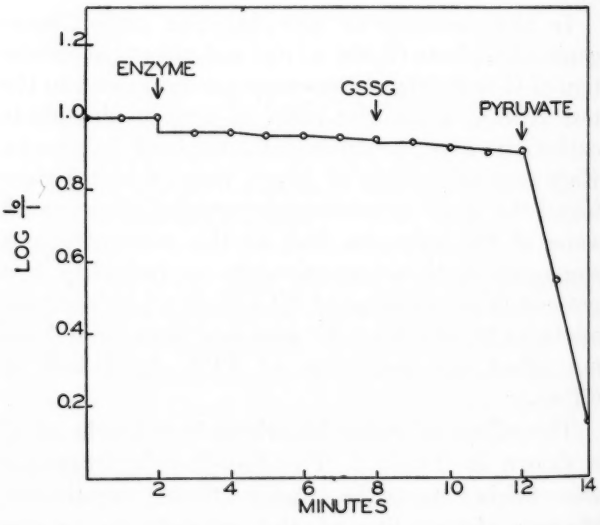

CHART 4.—Absence of DPN-GSSG reductase in ascites tumor extract. Initial volume, 2.9 cc., containing 100 μM TRIS pH 7.4 and 0.46 μM DPNH. At first arrow, 0.1 cc. enzyme added (acetone powder extracted with 0.1 M glycylglycine pH 7.4). At second arrow, 0.1 cc. GSSG (5 μM) added. At third arrow, 0.02 cc. sodium pyruvate (5 μM) added. Blank cells contained all components except DPNH. Temperature, 26° C. Wave-length, 340 mμ.

DISCUSSION

The considerable rates of oxidation of G-6-P and 6-P-G by ascites tumor extracts suggests that the direct oxidation of G-6-P via a TPN specific system may contribute to the over-all respiration of this tumor, especially in the presence of added sugar. The fact that glucose was unable to reduce pyridine nucleotides under conditions where vigorous reduction of TPN by G-6-P and 6-P-G occurred suggests that glucose dehydrogenase is not present in the ascites tumor cell. The degree of participation of the "hexose monophosphate shunt" in the respiration of the ascites tumor cannot, however, be surmised from these experiments. A study of the relative rates of combustion of glucose labeled at various carbon atoms with isotopic carbon by intact ascites tumor cells is indicated to answer this question.

TABLE 3

G-6-P AND 6-P-G DEHYDROGENASE AND ENOLASE ACTIVITY OF ASCITIC PLASMA

SAMPLE	PROTEIN CONTENT (PER CENT)	μM SUBSTRATE CHANGED/10 MIN/CC OF ASCITIC PLASMA		
		G-6-P	6-P-G	2-P-G
1	2.9	0.060	0.078	
2	3.7	0.065	0.137	2.70

Reactions were carried out in a final volume of 3 cc. at 25° C. Blank cells contained all components except the substrate. G-6-P and 6-P-G oxidation measured with 0.2–0.6 cc. ascitic plasma in the presence of glycylglycine pH 7.4 (100 μM); $MgCl_2$ (20 μM); 21 μM G-6-P; 1.5 μM 6-P-G; and 0.35 μM TPN. Reaction followed by increase in optical density at 340 mμ. Enolase activity measured with 0.03 cc. ascitic plasma with sodium phosphate pH 7.4 (100 μM); $MgCl_2$ (30 μM) and 40 μM 2-P-G; change in optical density measured at 240 mμ.

SUMMARY

Extracts of Ehrlich ascites tumor cells were shown to contain active glucose-6-phosphate and 6-phosphogluconate dehydrogenases which are specific for triphosphopyridine nucleotide. The activity of these enzymes is of the same order of magnitude as that of the isocitric dehydrogenase of these cells, but considerably less than that of the lactic dehydrogenase. No evidence for the presence of either glucose dehydrogenase or pyridine nucleotide transhydrogenase was obtained. Ehrlich ascites tumor cells possess powerful glutathione reductase activity.

Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities were also demonstrated in the ascitic plasma.

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Transplantation of the Shope Papilloma and the Rous Sarcoma during Early Developmental Stages*

HARRY S. N. GREENE

(Department of Pathology, Yale University School of Medicine, New Haven, Conn.)

Serial transplantation experiments undertaken at various stages in the development of spontaneous animal tumors indicated that the ability to grow on transfer to normal unrelated animals was a developmental acquisition and not a property of the tumors from their inception (3). Transplantability of this order appeared to be coincidental to the attainment of the ability to metastasize and extended to heterologous as well as homologous species. Heterologous transplantation experiments involving human tumors disclosed the occurrence of comparable developmental phases and suggested the existence of a similar relationship between transplantability and metastasizability (4). However, in the course of this study a striking exception in respect to behavior was found to characterize the transplantation reactions of four human tumors. These were the meningiomas, the Schwannomas, the mixed salivary gland tumors, and the bladder papillomas. Such tumors were transplantable from the time of clinical discovery, and the ability to grow in normal animals bore no relationship to metastasizability. Among animal tumors, the Shope rabbit papilloma and the Rous chicken sarcoma have also been found to be anomalous in this respect, and both tissues possess the ability to grow in homologous and heterologous hosts at periods of development long antedating metastasis (1, 5).

In past experiments the homologous and heterologous transplantation of the two animal tumors was successfully effected with tissue obtained at various times during their early course, and no attempt was made to determine whether or not the property of transplantability was present from their initiation. Investigation of this question was expedient, for the absence of a nontransplantable phase would constitute a significant variation from the biological history of other tumors studied in this laboratory and would apply directly to a conception of cancer autonomy as a developmental state. Data relative to the human tumors was not applicable to the problem, and human material adequate for a pertinent study was obviously unobtainable. Accordingly, an investigation of the animal tumors with respect to the inception of transplantability was instituted, and the results obtained are described in the present report.

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MATERIALS AND METHODS

The tumors utilized were induced by inoculation of domestic rabbits and chickens with cell-free filtrates of the Shope papilloma and the Rous sarcoma. The region of inoculation was biopsied and tissue obtained for transfer at intervals prior to and immediately following the appearance of lesions.

The tissue obtained from rabbits inoculated with the Shope papilloma virus was transplanted to other unrelated domestic rabbits and to guinea pigs, hamsters, and mice. Chicken tissue was transferred to rabbits, guinea pigs, and mice, but homologous hosts were not used, for the connective tissue present in the chicken's brain reacts to the Rous virus with the production of sarcomas, and a distinction from successful transplants cannot be made with certainty. The brain was used as a transplantation site in all cases. The technic employed has been described in detail (2).

RESULTS

Shope papilloma.—The filtrate was derived from glycerinated cottontail papilloma tissue known to produce visible changes in the scarified skin of domestic rabbits in from 18 to 20 days, and in order to study early lesions transplantation experiments were begun on the 14th day. Further biopsy specimens were transplanted to normal animals on the 16th and 21st days, and the series was

controlled by the transfer of normal scarified skin obtained prior to treatment with the virus.

The transplantation of normal, uninfected skin resulted in takes in rabbit brains, but the tissue failed to survive transfer to the brains of heterologous species. At the end of a month the homologous transplants had increased in size and were well vascularized. Both dermis and epidermis were histologically intact. Mitoses were common in the lower layers of the epidermis and in hair follicles, and formed hairs were occasionally present. Epithelial-lined cysts filled with keratin were found in many transplants, but changes comparable to those characteristic of the Shope papilloma were absent (Figs. 1, 2).

Biopsy specimens obtained on the 14th day after treatment showed microscopic, scattered areas of thickened epidermis with early hyperkeratotic changes of the nature found in typical papillomas, and transplantation of the tissue was successfully effected in both homologous and heterologous species. Characteristic tumors were found in the brains of six of the thirteen rabbits used. The tumor masses were comparable in size to those resulting from the transfer of fully developed papillomas and frequently occupied the greater part of a cerebral hemisphere. Histologically, they were composed of papillomatous epidermoid epithelium and keratin (Fig. 3). The transplants in four of the remaining seven rabbits consisted of normal adult skin without papillomatous changes, and it seems probable, in these cases, that infected cells were not included in the fragments used for transfer.

The brains of guinea pigs and mice were used as sites for heterologous transplantation. No takes occurred in the twelve guinea pigs used, but twelve of the twenty mice bore growths (Fig. 4). These were larger in relation to brain size than the corresponding tumors in rabbits and in a number of instances resulted in death of the mouse in less than a month after transfer.

On the 16th day after scarification, a slight papular eruption was apparent on examination with a hand lens, and histological examination showed, in minor degree, the epithelial proliferation characteristic of the fully developed papilloma. Transfer was successful in the six rabbits used and in eight of the ten mice, but again there were no takes in guinea pigs.

Minute papules were visible to the naked eye on the 21st day, and individual lesions were dissected free of adjacent skin for transplantation. Transfer was made to the brain of rabbits and to the brain and subcutaneous space of guinea pigs, mice, and hamsters. No takes occurred in the subcutaneous space of guinea pigs, but large growths were present in the brain at the end of a month and in both sites in the other species (Figs. 5–9).

Rous sarcoma.—A cell-free filtrate of the Rous sarcoma was inoculated in the breast muscle of young chickens which were killed at 8, 10, and 12 days to provide material for transfer. Microscopic examination of tissues obtained on the 8th and 10th days showed foci of growth, but the tumor was not sufficiently demarcated to allow a clean separation from adjacent muscle before the 12th day. Accordingly, the material used for transfer on the 8th and 10th days consisted largely of random fragments of muscle from the inoculation site, while that employed on the 12th day was composed solely of tumor.

The brains of rabbits, guinea pigs, and mice were used as sites for transplantation, and the animals were killed 10 days after transfer. Microscopic examination of the transplants prior to the 12th day showed degenerating muscle surrounded by a zone of inflam atory reaction, and there was no evidence of tumor growth. However, the transplants of the 12th day were made up of characteristic sarcoma tissue with little or no evidence of foreign body reaction and had increased by 2–8 diameters in size. Takes occurred in all the six rabbits used, in eight of the twelve guinea pigs, and in eleven of the fifteen mice (Figs. 10–16).

DISCUSSION

It is apparent from these results that the Shope papilloma and the Rous sarcoma possess the ability to survive and to grow on homologous and heterologous transfer from their earliest stages of development. This property is not an attribute of other tumors at corresponding periods but, on the contrary, is a late development coincident with the attainment of metastasizability and antecedent to a fatal termination. Such associations are not found in the cases of the Shope and Rous tumors. Transplantability is present from inception, yet metastasizability is only acquired after an extended developmental course.

An investigation of transplantation reactions in the pre-metastasizable phase of the development of ordinary tumors suggests that the failure of growth in normal animals relates to a dependence on factors peculiar to the tumor-bearing animal (3). Such factors are not supplied by normal animals but are shared by the primary host and other animals bearing spontaneous tumors of the same nature. They are present from the earliest stages of tumor development and appear to be constitutional in nature. The early Shope and Rous tumors are of special interest from this point of view, for

their transplantability suggests either that they are autonomous from inception or that the factor on which their continued existence depends is not constitutional in nature but is transmitted to the new host along with the tumor cell.

Both the Shope papilloma and the Rous sarcoma are virus-induced tumors, and, during the greater part of their course, their continued growth appears to be conditioned on the continued presence of the virus. The virus is intracellular in location and necessarily accompanies the tumor cell in its transfer to a new host. It is suggested that the intracellular virus is analogous in its operation to the constitutional factors concerned in the dependence of other tumors and that the transplantability and growth of the Shope papilloma and the Rous sarcoma in normal animals is conditioned on the concomitant transfer of their dependent factors. The attainment of autonomy, or independence of conditioning factors, is a final stage in the development of other tumors, and there is evidence of a parallelism in the course of the Shope papilloma; for in the final stage of epidermoid carcinoma, its independence of conditioning factor is manifest by growth and transplantability in the absence of detectable virus (6–8).

The latter point is of some interest, as it may apply to investigations concerned with the possible virus etiology of other tumors. If the virus operates as a conditioning factor in such instances and the attainment of independence of the factor is a sequence in the natural development of the tumor, it is possible that, as in the case of the Shope papilloma, the virus may disappear with the assumption of autonomy. Accordingly, tumors in early or dependent phases of development would constitute a more logical material for virus search than the fully evolved, autonomous cancer.

A conception of the virus as a conditioning factor analogous in its action to the constitutional factors concerned in the dependence of other tumors and located in the cell rather than in the host implies that transfer of an infected cell would necessarily be accompanied by transfer of the factor on which its continued growth depends. Thus, the special environmental conditions requisite for the growth of other tumors transplanted during dependent phases of development are replaced by intracellular components in the case of virus tumors, and in this respect the survival and growth of transplants are independent of the constitutional status of the host. Accordingly, a question of a virus etiology is associated with tumors capable of growth in normal unrelated hosts during the dependent or pre-metastasizable phase of development and applies to the four human tumors characterized by such behavior—the meningioma, the Schwannoma, the mixed salivary gland tumor, and the bladder papilloma.

SUMMARY

Unlike other tumors studied, the Shope papilloma and the Rous sarcoma are heterotransplantable from their inception. The significance of this finding is discussed.

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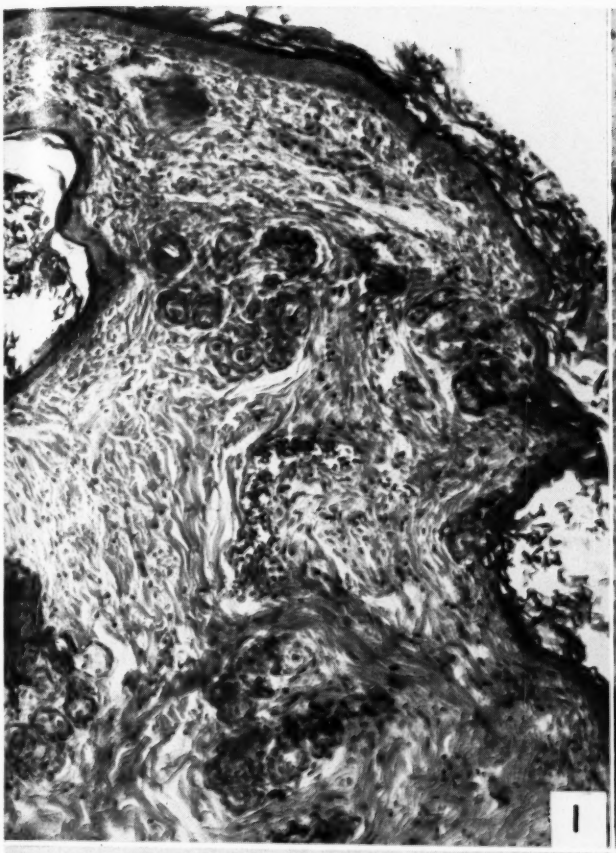
Fig. 1.—Transplant of fragment of normal adult rabbit skin in the brain of a normal rabbit killed 1 month after transfer. The fragment used for transfer was obtained by biopsy prior to scarification and application of the Shope virus. ×175.

Fig. 2.—Transplant of fragment of normal adult rabbit skin in the brain of a normal rabbit killed 1 month after transfer. The fragment used for transfer was obtained by biopsy of a scarified area prior to treatment with the virus. ×175.

Fig. 3.—Brain transplant of adult rabbit skin obtained by biopsy of a scarified area 14 days after treatment with the Shope virus. The rabbit bearing the transplant was killed 30 days after transfer. ×35.

Fig. 4.—Transplant of adult rabbit skin obtained by biopsy of a scarified area 14 days after treatment with the Shope virus growing in the brain of a DBA mouse. The mouse was killed 27 days after transfer. ×175.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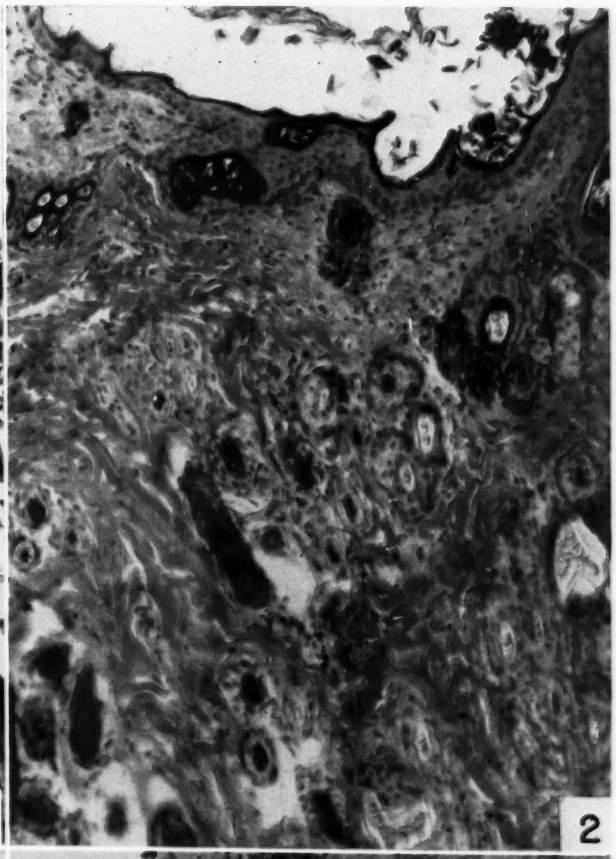
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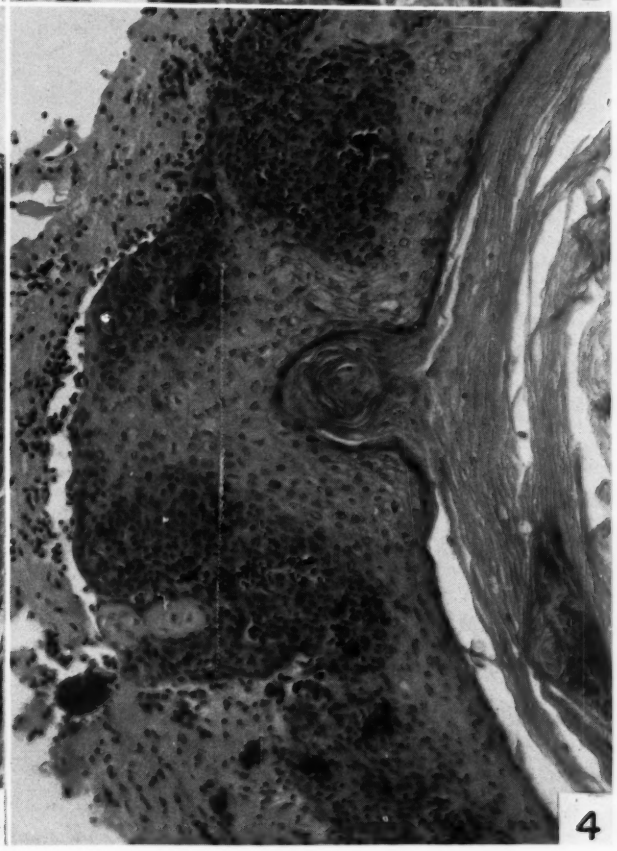
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FIG. 5.—Transplant of early papilloma, growing in the brain of a DBA mouse. The fragment used for transfer was obtained by biopsy from the skin of a rabbit 21 days after application of the Shope virus. The mouse bearing the transplant was killed 24 days after transfer. ×175.

FIG. 6.—Transplant of early papilloma growing in the brain of a guinea pig. The fragment used for transfer was obtained by biopsy from the skin of a rabbit 21 days after application of the Shope virus. The guinea pig was killed 31 days after transfer. ×175.

FIG. 7.—Transplant of early papilloma growing in the brain of a hamster. The transplanted fragment was obtained from the skin of a rabbit 21 days after treatment with the Shope virus and the hamster killed 32 days after transfer. ×175.

FIG. 8.—Transplant of early papilloma growing in the subcutaneous space of a DBA mouse. The transplanted fragment was obtained from the skin of a rabbit 21 days after treatment with the Shope virus, and the mouse was killed 33 days after transfer. ×175.

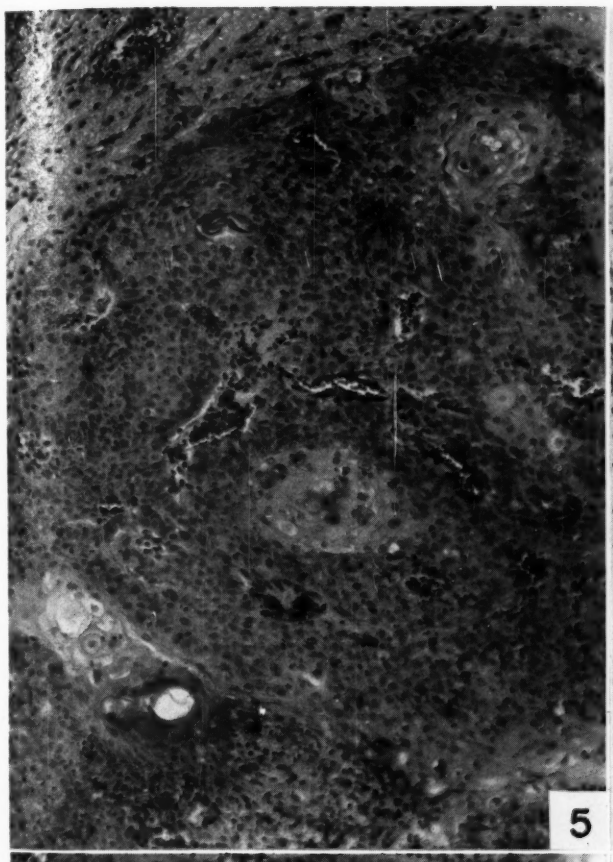
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FIG. 9.—Transplant of early papilloma growing in the subcutaneous space of a hamster. The transplanted fragment was obtained from the skin of a rabbit 21 days after application of the virus and the hamster killed 58 days after transfer. ×40.

FIG. 10.—Transplant of Rous chicken sarcoma in the brain of a rabbit. The tissue was obtained from a chicken 12 days after injection of the virus, and the rabbit was killed 10 days after transfer. Note area of chicken muscle included in transplanted tissue. The muscle is dead and necrotic in contrast to actively growing tumor. ×85.

FIG. 11.—Transplant of Rous chicken sarcoma in rabbit's brain. The tumor was obtained from a chicken 12 days after injection of the virus, and the rabbit was killed 10 days after transfer. ×320.

FIG. 12.—Transplant of Rous chicken sarcoma in a guinea pig's brain. The tumor was obtained from a chicken 10 days after injection of the virus, and the guinea pig was killed 10 days after transfer. ×85.

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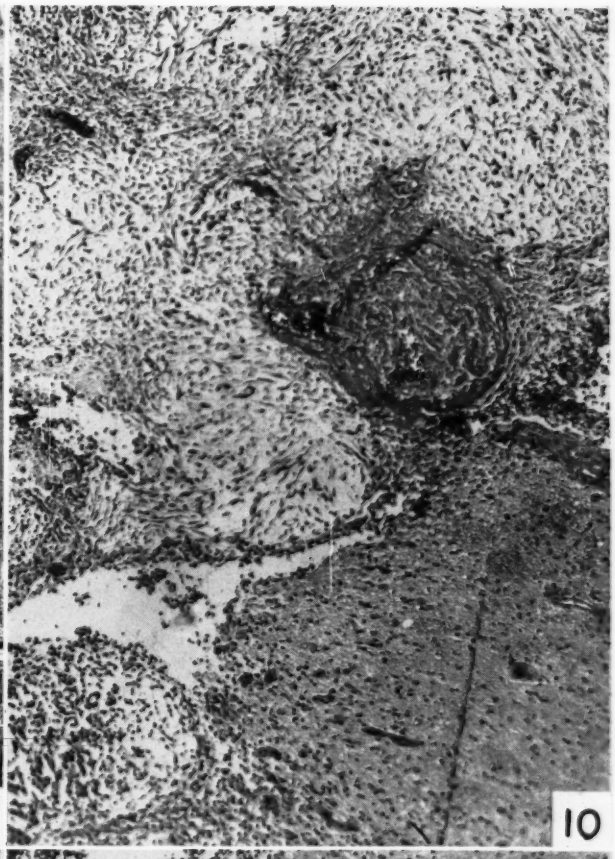

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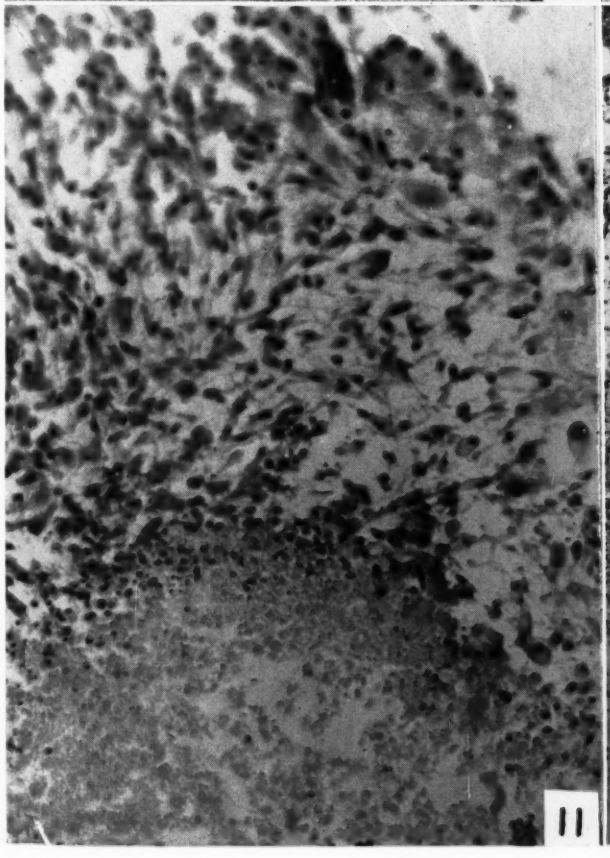

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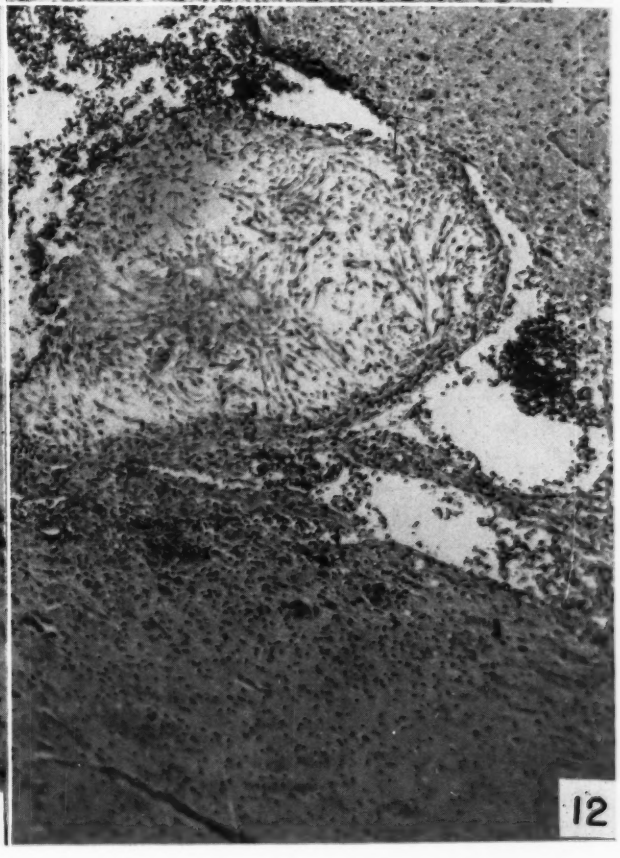

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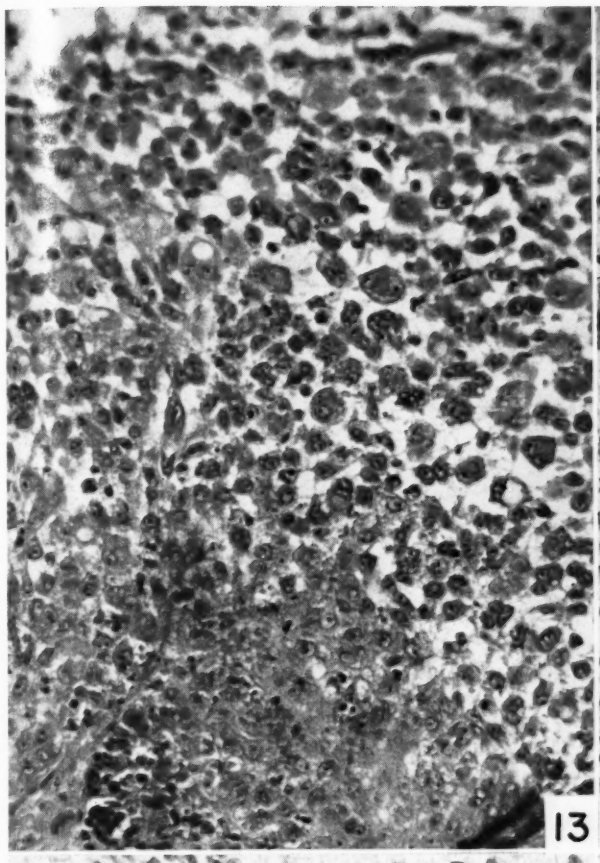
FIG. 13.—Transplant of Rous chicken sarcoma in a guinea pig's brain. The tumor was obtained from a chicken 10 days after injection of the virus, and the guinea pig was killed 10 days after transfer. ×320.

FIG. 14.—Transplant of Rous chicken sarcoma in the brain of a DBA mouse. The tumor was obtained from a chicken 10 days after injection of the virus, and the mouse was killed 10 days after transfer. ×230.

FIG. 15.—Transplant of Rous chicken sarcoma in the brain of a DBA mouse. The tumor was obtained from a chicken 10 days after injection of the virus, and the mouse was killed 10 days after transfer. ×320.

FIG. 16.—Transplant of Rous chicken sarcoma in the brain of a DBA mouse. The tumor was obtained from a chicken 10 days after injection of the virus, and the mouse was killed 10 days after transfer. ×320.



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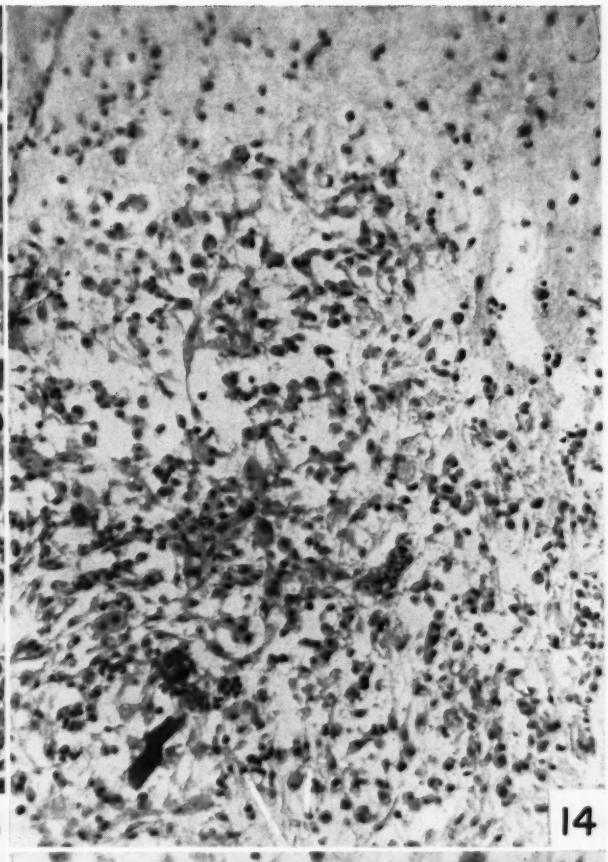

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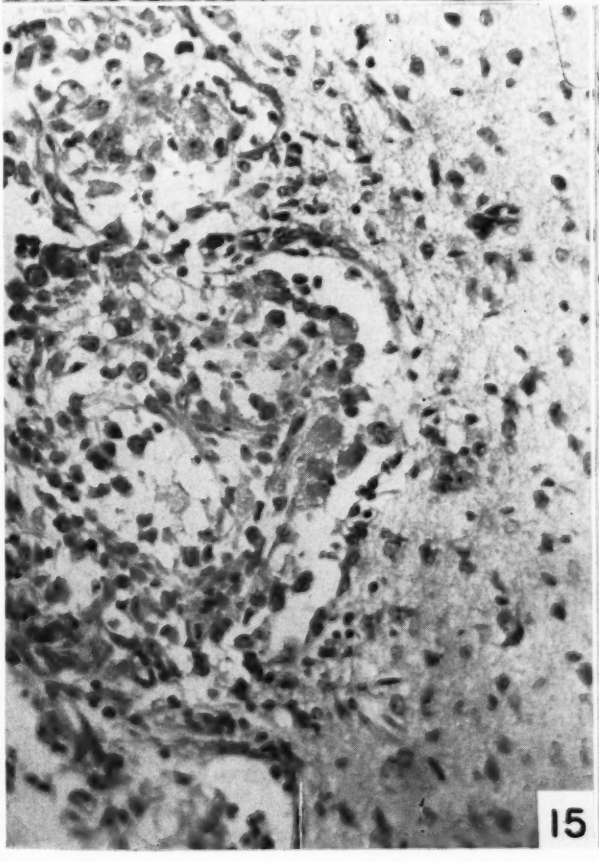

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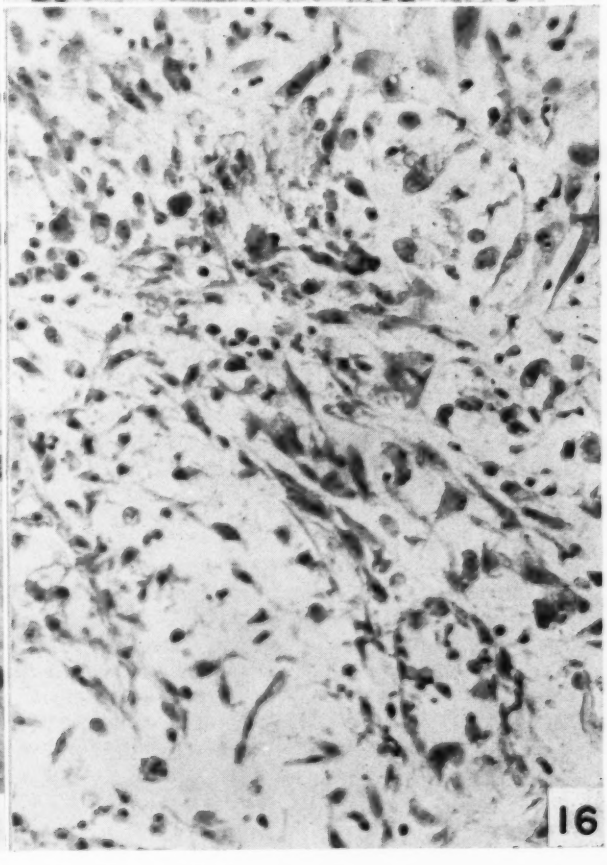

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Nucleic Acid Content of Splenic Lymphocytes in Normal and Leukemic Mice*

M. L. Menten, M. Willms, and W. D. Wright

(*British Columbia Medical Research Institute, Vancouver, British Columbia*)

In a plan to study the consecutive changes which the nucleic acids of a cell may undergo under varying experimental conditions, the preliminary problem which confronted us was the production of a uniform suspension of cells. After many attempts to produce such a suspension from the different tissues of the C57 strain of black mice, it became manifest that only with spleen and a transplanted mammary tumor could a sufficient number of discrete intact cells for enumeration and chemical analyses be obtained in a fluid medium. Data on cellular desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) of splenic lymphocytes are presented in this paper.

Few chemical determinations on nucleic acid content per cell have been reported on cellular suspensions of normal mammalian tissue other than bone marrow (3) and neutrophils (8) because of the difficulty in producing, from compact tissues and organs, a cellular suspension free from clumps of cells. However, there have been recorded estimates of the DNA content of cells obtained by chemical analysis on isolated nuclei, because DNA is limited to the nucleus and this nucleic acid is not readily disintegrated. Petermann and Schneider (11, 12) reported 6.5×10^{-9} mg. of DNA in nuclei of lymphocytes isolated from the spleen of normal Akm mice. In these mice bearing transplanted lymphatic leukemia, the nuclear content of DNA was 9.4×10^{-9} mg., an increase of almost 50 per cent. Vendrely and Vendrely (14) found a value for DNA of 6.4×10^{-9} mg/nucleus for cells of calf thymus. Davidson (4) recorded a similar figure of 7.5×10^{-9} mg., and Mirsky and Ris (10) found 7.1×10^{-9} mg. of DNA for these cells. The latter investigators also obtained 7.03×10^{-9} mg of DNA/nucleus for lymphocytes from calf lymph node. Swift (13), making photometric determinations on Feulgen preparations of various mouse tissues, found a considerable range of values for cellular DNA. Lymphocytes from spleen of this animal gave a DNA value of 3.12×10^{-9} mg/cell, but these were arbitrary spectrophotometric units. Leuchtenberger, Klein, and Klein (6), however, have made comparisons of microphotometric and biochemical findings on nuclei of lymphocytes in an ascites lymphoma of DBA mice and with either technic reported almost identical DNA values which nearly duplicate the figure of 6.5×10^{-9} mg. reported by Petermann and Schneider (11, 12) for the nucleus of the lymphocyte of normal mouse.

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MATERIALS AND METHODS

Spleens from normal C57BL mice, as well as from those inoculated subcutaneously with cells of a granulocytic leukemia, were used. The granulocytic leukemia, C1498, was procured from the Roscoe B. Jackson Laboratory at Bar Harbor, Maine. Also included for comparison were two spleens removed from healthy dogs following experimental surgery and sections from two human spleens obtained at necropsy performed 2 hours post mortem. The human spleens were of normal weight and microscopically free from demonstrable pathologic lesions. Analysis of lymphocytes derived from one of these was made on nuclei only, and, in the second case, analyses were made on whole cells before and after exposure at 20° C. The suspensions were prepared by rapidly grinding freshly extirpated spleens to which was added 0.5 cc. of 0.1 M citric acid in a Potter and Elvehjem glass homogenizer. Later, the cells were separated with less apparent trauma by rapid grinding with a pestle in a small glass mortar with Ringer's solution as a diluent. Several mouse spleens were pooled in order to obtain a heavy suspension which was filtered and refiltered through glass wool under reduced pressure until a cellular filtrate free from clumps of cells was attained. Usually, two filterings sufficed to produce a uniform cell suspension, as shown by rapid microscopic examination on a slide of a mixture of a drop of filtrate with a drop of stain. Enumeration of cells in a Neubauer hemocytometer and the ensuing chemical procedures were similar to those described previously (9). The effect of 24 hours' incubation at 20° C. on the cellular nucleic acid was studied in two filtrates, one containing mouse lymphocytes and the other human lymphocytes. This assay was suggested by the not infrequent association of varying values of cellular DNAP and RNAP obtained in different lots of lymphocytic suspensions.

RESULTS

The figures for cellular DNAP and RNAP and the ratio of RNAP to DNAP for lymphocytes derived from various spleens are listed in Tables 1

and 2. The data in Table 1 were derived from normal mouse lymphocytes and the data in Table 2 from lymphocytes of leukemic mouse, man, and dog. The ratio of RNAP to DNAP falls into a series of groups each with a relatively well defined value. These ratios form, in ascending order, the basis for the arrangement of the tabulated data.

TABLE 1

CONTENT OF NUCLEIC ACID PHOSPHORUS PER SPLENIC LYMPHOCYTE OF NORMAL MOUSE

DNAP mg.×10^{-9}	RNAP mg.×10^{-9}	RNAP/DNAP
0.54	0.16	0.30
0.55	0.17	0.31
0.46	0.17	0.37
0.48	0.18	0.37
0.48	0.18	0.37
0.48	0.18	0.37
0.53	0.19	0.36
0.56	0.21	0.37
0.57	0.21	0.37
0.47	0.21	0.45
0.47	0.22	0.47
0.49	0.23	0.47
0.37	0.20	0.54
0.42	0.23	0.53
0.48	0.26	0.54
0.31*	0.14	0.45

* After 24 hours' incubation at 20° C. of previously listed suspension.

TABLE 2

CONTENT OF NUCLEIC ACID PHOSPHORUS PER SPLENIC LYMPHOCYTE OF LEUKEMIC MOUSE, DOG, AND MAN

DNAP mg.×10^{-9}	RNAP mg.×10^{-9}	RNAP/DNAP
	Leukemic mouse	
0.66	0.29	0.44
0.52	0.28	0.54
0.56	0.31	0.55
0.66	0.37	0.56
0.72	0.49	0.68
	Dog (post surgical)	
0.60	0.34	0.56
0.57	0.39	0.68
	Human (2 hours post mortem)	
0.64	0.20	0.31
0.56*	0.17	0.30
0.59 (Nuclei)	0.09	0.15

* After 24 hours' incubation at 20° C. of previously listed suspension.

In Table 1, these values are 0.30, 0.37, 0.45, and 0.55; and, similarly, in Table 2, 0.31, 0.45, 0.55, and 0.68. Within each group the cellular DNAP values are also aligned in ascending order, with two exceptions. Individual figures for cellular DNAP and RNAP are recorded in the tables and each pair of these nucleic acids has been plotted graphically in Chart 1, to show possible relationships, one to the other.

It may be noted (Table 1) that the range of values for both DNAP and RNAP in lymphocytes of normal mice was wide, being from 0.37 to 0.56 × 10^{-9} mg. (50 per cent spread) for the cellular DNAP and from 0.16 to 0.26 × 10^{-9} mg. (60 per cent spread) for the cellular RNAP. The mean values per cell were 0.49 × 10^{-9} mg. and 0.20 × 10^{-9} mg., respectively. Splenic lymphocytes from mice with subcutaneous implants of myelogenous leukemia contained a greater amount of both nucleic acids, with a range from 0.52 to 0.72 × 10^{-9} mg. and a mean of 0.61 × 10^{-9} mg/cell for DNAP, and a range from 0.28 to 0.50 × 10^{-9} mg. with a mean of 0.37 × 10^{-9} mg/cell for RNAP (Table 2). The increase in mean lymphocytic DNAP of the leukemic (even though myelogenous in type) animal over the mean cellular DNAP of the lymphocyte of the normal mouse was 24 per cent and for RNAP, 85 per cent. Although the number of analyses on species other than mouse was small, in man and dog (the two different species studied), the DNAP averaged 0.60 × 10^{-9} mg. and the RNAP 0.28 × 10^{-9} mg/lymphocyte.

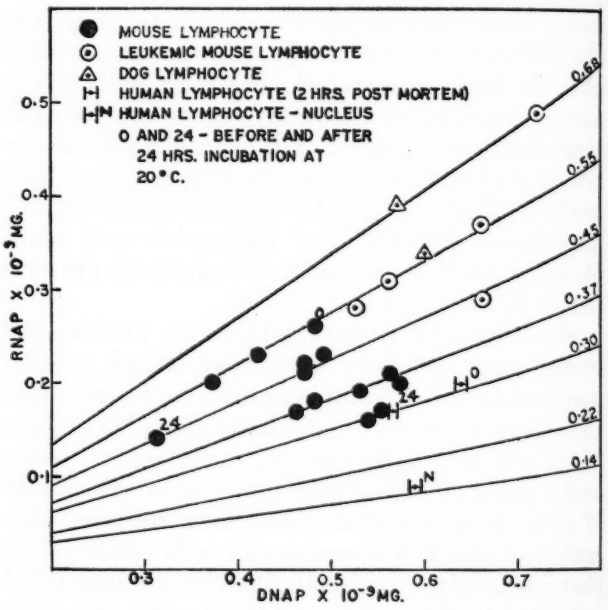

CHART 1.—Relationship of RNAP to DNAP in splenic lymphocytes of normal and leukemic mouse, dog, and man.

In Chart 1, the paired cellular values of DNAP and RNAP, obtained chemically and listed in Tables 1 and 2, were plotted in a scatter diagram in which the abscissa x was cellular DNAP and the ordinate y was cellular RNAP. The majority of the plotted points in the scatter diagram fell into a

series of "lines of regression," each of which, when projected downward and to the left, ended in zero. The slope of these various lines may be expressed by $y = a + bx$, the formula for a straight line. Because the y intercept "a" is absent or 0, the formula becomes $y = bx$ where "b" is the coefficient of x and a constant. The constant "b" is the ratio of RNAP to DNAP. The value of "b" in the five lines of regression is in sequence 0.30, 0.37, 0.45, 0.55, and 0.68. As the lines approach the perpendicular, the RNAP value increases.

DISCUSSION

The mean DNA value (approximately 10 times that of the DNAP) obtained by us for the mouse lymphocyte is lower than that recorded by Petermann and Schneider (11, 12) and by Vendrely and Vendrely (14) for the nuclei of these cells. A possible explanation for this difference may lie in the less drastic manipulation to which splenic tissue was subjected in the preparation of cellular filtrates by us in comparison to the treatment required for the production of a suspension of nuclei. The lymphocyte lying in the splenic sinuses ready to be delivered into the circulation has undoubtedly reached the late phase of its existence, a state probably beyond its maximal maturation, as disclosed by pyknosis of its nucleus. Pyknosis, evidence of depolymerization of nuclear material (5), attests its ephemeral survival, which has been computed at 3 hours after its entrance into the blood stream. Increased manipulation tends to eliminate the aging and less resistant cell types, which may be preserved by a less traumatic handling. The dog lymphocytes analyzed by us had been subjected *in vivo* to untoward conditions arising from operative procedures; the human material has suffered degradation from post mortem environment; the more degraded lymphocytes have been eliminated, and our values for the remaining lymphocytes approach those recorded for nuclei in the literature. One consideration may be that only cells in the free state were selected for analysis and the others were discarded. The lowest DNAP values obtained by us approximate those reported by Swift (13).

The most patent feature displayed in Chart 1 is the small area occupied by the splenic lymphocytes of the normal mouse, in comparison to the greater space over which the remaining lymphocytes are distributed. A second feature is the relatively high RNAP of the lymphocyte of the dog and of the mouse inoculated with myelogenous leukemia, one indication of which is the unique position of two of these points on the line of regression in which the constant "b" reaches 0.68. The higher level of nucleic acids in splenic lymphocytes derived from mice inoculated with granulocytic leukemia may have been due to inclusion in the splenic sinusoids of metastatic myelocytic cells and their being freed on grinding. It was not possible to differentiate lymphocytic and myelocytic cells in our stained films of splenic filtrates. Spleen was the exception to the finding of Cerecedo *et al.* (1, 2, 7) that the levels of the nucleic acids tended to be increased in different organs of tumor-bearing animals. The effect of altered environmental conditions is shown by the devolution of both RNAP and DNAP, which exposure for 24 hours at 20° C. produced once in the normal mouse and once in the human lymphocyte.

These two single instances of depolymerization of cellular nucleic acids in catabolic processes are included here. Evidence, with amplification of detail, for a similar phenomenon will be reported in a later paper.

SUMMARY

1. The DNAP content per splenic lymphocyte of normal mouse spleens varied between 0.37×10^{-9} and 0.57×10^{-9} mg., with a mean value of 0.49×10^{-9} mg. This mean value is lower than that reported for the DNAP of the isolated nucleus of the mouse lymphocyte. Possible reasons for the difference are discussed. The RNAP content per lymphocyte varied between 0.16×10^{-9} and 0.26×10^{-9} mg., with a mean value of 0.20×10^{-9} mg.

2. The DNAP of splenic lymphocytes from leukemic mice varied between 0.66 and 0.72×10^{-9} mg. The RNAP varied between 0.29 and 0.49×10^{-9} mg. Possible reason for higher values in leukemic mice is briefly discussed.

3. Relationship of RNAP to DNAP has been shown graphically by plotting values of the two nucleic acids obtained for splenic lymphocytes of both normal and leukemic mice in a scatter diagram. From the plot five lines of regression were developed with coefficients of correlation with values of 0.30, 0.37, 0.45, 0.55, and 0.68. Also included are nucleic acid analyses on lymphocytes derived from two dogs and two human spleens. Inclusion of values of human lymphocytes adds a lower line of regression with a coefficient of correlation of 0.14. It is postulated that these values represent phases in the relationship of RNAP to DNAP under varying conditions within the cell.

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Nucleic Acid Content of Cells of Bone Marrow Aspirated from Patients with Leukemia*

M. L. Menten and Mary Willms

(British Columbia Medical Research Institute and the Vancouver General Hospital, Vancouver, British Columbia)

This report deals with the cellular content of ribonucleic acid and desoxyribonucleic acid in 33 samples of bone marrow aspirated from 30 patients with leukemia. Of the latter number, fourteen were diagnosed as cases of granulocytic leukemia and sixteen as lymphocytic leukemia. The majority of the patients were adults seen in the Vancouver General Hospital and the British Columbia Cancer Institute. A few patients in the Shaughnessy Veterans' Hospital and the Royal Columbia Hospital, New Westminster, were also made available to us. Patients with and without treatment have been included.

Comparatively few results of chemical analysis of the nucleic acid content per cell of suspensions of bone marrow have been found in the literature. The fairly uniform distribution of discrete cells following dilution of this tissue in media such as normal saline or Ringer's solution, however, readily allows enumeration of cells and evaluation of their chemical constituents. Davidson, Leslie, and White (1) analyzed bone marrow from seventeen patients with leukemia, fifteen of which were granulocytic in type, but these authors report an average value per cell for the seventeen cases.

METHODS

Procedures for obtaining samples of bone marrow and the technic used in their chemical analyses were identical with those outlined in a previous paper (6). Values obtained are expressed as desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP). Cell counts on samples of the bone marrow suspensions studied were in most experiments checked by a second person. Suspensions containing a small number of cells tended to give wider variations in values for nucleic acid than those containing a high number of cells, mainly because of difficulty in obtaining precise colorimetric readings for low phosphorus content. Also, slight inaccuracies in the enumeration of cells in a suspension containing a low count/c mm. may markedly alter the values obtained on computation of nucleic acid/cc. Goldberg, Klein, and Klein (2) have discussed possible variations arising in the cell count in suspensions of ascites tumor cells. A factor occasionally influencing the cell count was found to be the presence of minute discrete clots in the original bone marrow sample which sometimes occurred in imperfectly siliconed tubes. At the time of analysis, these clots were deemed too small in size and number to induce spontaneously any considerable removal of cells from the bone marrow samples, such as occurs with larger sized coagula. These latter, by entrapping cells, may considerably lower the count/c mm(6) and, consequently, increase the value of nucleic acid per cell obtained analytically. Stained films of bone marrow from patients studied in the Vancouver General Hospital were kindly loaned to us from the Department of Hematology. When patients were seen in other hospitals, films of bone marrow were made and stained by us.

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RESULTS

The results of the chemical analyses performed on leukemic bone marrow are presented in Tables 1 and 2. The figures obtained per cell in lymphocytic leukemia are listed in Table 1 and values found for granulocytic leukemia in Table 2. The data are arranged with the DNAP per cell in ascending order, with the exception of the second entry for J.P. in Table 1. The level of cellular nucleic acid in these leukemic patients showed considerable variation, especially in those who had received x-radiation. Following x-radiation, both DNAP and RNAP showed a decrease, but the latter to a slightly greater extent, as is seen in the first six entries in Table 1. Reduction of the nucleic acid content was marked when the dosage of x-ray was high, when treatment was carried out over a long period, and also when only a short time had elapsed between the last exposure to radiation and the aspiration of the marrow. An example of the latter condition is indicated for J.P. in Table 1. In lymphocytic leukemia, the values for DNAP per cell ranged from 0.25 to 1.00×10^{-9} mg. and in granulocytic leukemia from 0.36 to 0.90×10^{-9} mg. The DNAP values for B.L. and A.B. in Table 1 seem unusually high and may have been due to an abnormally low initial cell count/c mm. Furthermore, in the bone marrow of these two patients, the cellular RNAP is low in comparison to the high DNAP; in our experience, high DNAP has been commonly associated with a high RNAP, especially in the blastic

TABLE 1

Content of Nucleic Acid Phosphorus per Cell in Bone Marrow Aspirated from Patients with Lymphocytic Leukemia

Patient	Age (years)	DNAP ($mg \times 10^{-9}$/cell)	RNAP ($mg \times 10^{-9}$/cell)	RNAP/DNAP	Comments
M.C.	64	0.50	0.22	0.44	X-radiation until 6 weeks previously, slight enlargement of superficial lymph nodes.
T.G.	56	0.54	0.26	0.48	Leukemia of 3-yr. duration; x-radiation until 3 mos. prev.; splenomegaly; enlarged superfic. lymph nodes.
F.L.	71	0.55	0.37	0.67	X-radiation until 1 mo. prev. Leukemia blastic in type.
M.L.	60	0.55	0.31	0.56	X-radiation for 8 mos. ending 2 mos. prev. Leukemia chronic in type; rt. mastectomy 5 yrs. prev.
F.H.	50	0.61	0.18	0.30	O.P.D. patient with 3 days' treatment, type not specified. Moderate enlargement of superfic. lymph nodes.
S.R.	70	0.61	0.22	0.36	Urethan given until 2 wks. prev.; splenomegaly; hepatomegaly of 4-yr. duration; superfic. lymph nodes not palpable.
H.Y.	38	0.63	0.23	0.37	Folic acid given until 2 wks. and x-radiation until 3 mos. prev.
(10 days later)		0.64	0.23	0.36	
F.B.	75	0.65	0.23	0.36	Mediastinal mass; hypertrophy of prostate; superfic. lymph nodes enlarged; leukemia chronic in type.
A.V.	58	0.67	0.47	0.70	Spleen and superfic. lymph nodes not palpable; leukemia chronic in type.
J.P.	65	0.67	0.46	0.68	Splenomegaly; hepatomegaly; enlarged superfic. lymph nodes; x-radiation 1 yr. prev.
(2 wks. later)		0.25	0.11	0.44	X-radiation for prev. 2 wks.
D.K.	4	0.69	0.50	0.71	Leukemia chronic in type; not treated.
M.O.	72	0.70	0.50	0.70	Enlarged superfic. lymph nodes; leukemia chronic in type.
C.W.	76	0.70	0.59	0.84	Streptococcic otitis media; granuloma of left knee.
R.K.	57	0.72	0.62	0.86	X-radiation 3 mos. prev.; enlargement of rt. parotid and superfic. lymph nodes; splenomegaly.
B.L.	60	0.97	0.51	0.51	Superfic. lymph nodes enlarged; leukemia blastic in type.
A.B.	16	1.00	0.40	0.40	Splenomegaly; generalized enlarged superfic. lymph nodes. Leukemia blastic in type.

TABLE 2

Nucleic Acid Phosphorus per Cell in Bone Marrow Aspirated from Patients with Granulocytic Leukemia

Patient	Age (years)	DNAP ($mg \times 10^{-9}$/cell)	RNAP ($mg \times 10^{-9}$/cell)	RNAP/DNAP	Comments
G.G.	66	0.36	0.20	0.55	Urethan for 2 previous days.
V.H.	44	0.45	0.25	0.55	Splenomegaly; superfic. lymph nodes not palpable; leukemia chronic in type; x-radiation for several weeks prev.
C.M.	60	0.55	0.30	0.54	Questionable eosinophilia; x-radiation for several wks. prev.
(10 days later)		0.62	0.34	0.55	
W.W.	31	0.67	0.18	0.27	Urethan and x-radiation ending 12 wks. prev., then transfusion; splenomegaly; superfic. lymph nodes not palpable.
J.E.	83	0.67	0.45	0.67	Dyspnoea; hepatomegaly; duodenal ulcer prev. Leukemia chronic in type.
H.F.	64	0.78	0.20	0.26	Intestinal hemorrhage; petechiae of skin and buccal mucosa; leukemia blastic in type.
K.M.	73	0.78	0.35	0.45	Amitol and sulfadiazine for prev. 9 days.
B.G.	26	0.80	0.57	0.71	Edge of spleen palpable; leukemia blastic in type.
S.S.	59	0.81	0.45	0.55	Leukemia chronic in type.
S.F.	57	0.83	0.37	0.45	Aminopterin and ACTH with little improvement, died 1 mo. after aspiration of bone marrow.
B.A.	0.3	0.84	0.58	0.68	Leukemia blastic in type; splenomegaly; enlarged superfic. lymph nodes.
M.S.	51	0.88	0.42	0.48	Leukemia blastic in type.
C.C.	53	0.88	0.46	0.52	Leukemia blastic in type.
P.R.	73	0.90	0.42	0.47	Splenomegaly; superfic. lymph nodes not palpable. Hematemesis; urethan and x-radiation 9 mos. prev.

types of leukemia, the stained cells of which reveal considerable cytoplasmic basophilia. The range of RNAP in lymphocytic leukemia was from 0.18 to 0.62 $\times$ 10^{-9} mg/cell and in granulocytic leukemia from 0.20 to 0.58 $\times$ 10^{-9} mg/cell. For the major group of unirradiated patients with lymphocytic leukemia (or those for whom a period of at least 3 months had elapsed following irradiation), a mean figure of 0.74 $\times$ 10^{-9} mg. of DNAP/cell was obtained, or 0.68 $\times$ 10^{-9} mg. if the two doubtful higher values are excluded. For a similar group of patients with granulocytic leukemia, a mean value of DNAP of 0.82 $\times$ 10^{-9} mg/cell was obtained. In both types of leukemia, the RNAP/DNAP ratio ranged between 0.30 and 0.85. These "ratio" values suggested a correlation of DNAP and RNAP in leukemia analogous to that which we had obtained for lymphocytes. When the paired values for the two nucleic acids listed in Tables 1 and 2 were plotted as points in a scatter diagram on a "correlation graph" with DNAP $\times$ 10^{-9} mg. as abscissa and RNAP $\times$ 10^{-9} mg. as ordinate, lines of regression were developed with the following coefficients of correlation—0.28, 0.36, 0.45, 0.55, 0.69, and 0.85 (Chart 1). The observed range of individual ratios composing groups with the mean values underlying these listed coefficients of correlation is wider than that found for the lymphocyte (8). However, there seems no doubt that these listed average coefficients are common to both the leukemic and nonleukemic cell.

DISCUSSION

The question arises as to the relative accuracy of results established on a tissue which normally has such a mixed population of cells as bone marrow. In leukemia the cellular heterogeneity characteristic of normal marrow is gradually reduced by an influx of leukemic cells. In granulocytic leukemia these cells arise by hyperplasia of the marrow throughout the course of the disease; in the initial stages of lymphocytic leukemia, there is an infiltration of leukemic cells from outside the marrow and, in the advanced stages, a probable development of the primitive stem cells within the marrow directly into cells of lymphocytic leukemia. In most of our cases, stained films of leukemic marrow showed a preponderance of leukemic cells. Stained films of bone marrow aspirated from patients with acute leukemia showed a heterogeneity made up of differences in size, morphology, and structure of the anaplastic cells, which invariably had basophilic cytoplasm.

Contrary to the opinion previously expressed by one of us (M.L.M. [5]) that the anaplastic cells of acute leukemia contained a smaller amount of DNAP than the more mature cells of the chronic type of the disease, only a small difference in the amount of this constituent was found chemically, regardless of the type of cell predominating in the leukemia, provided the patient had not been irradiated. Actually, a trend toward a slightly greater amount of DNAP was shown for the more immature cell type, but cellular RNAP varied considerably. Reduction of DNAP, as well as the more labile RNAP cellular constituent, occurred following x-radiation of the patient but apparently

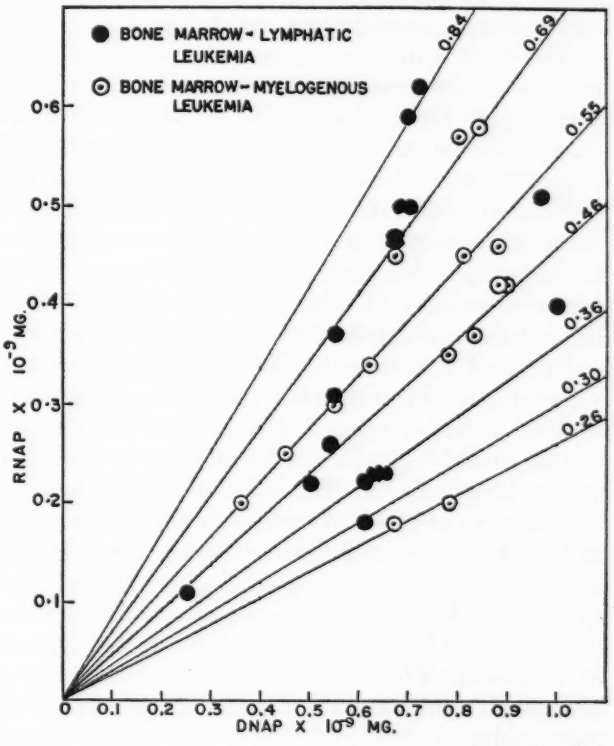

CHART 1.—Relationship of RNAP to DNAP in cells of bone marrow aspirated from patients with leukemia.

might also take place in a sample of bone marrow inadvertently exposed after aspiration to a slight rise in environmental temperature. This change may occur if the tube containing aspirated bone marrow is not immediately immersed in ice after withdrawal and maintained at 0° C. until analysis is begun. Reduction in nucleic acids due to temperature will be reported later.

It is of interest that the cellular content of DNAP found by us in human lymphocytic leukemia closely approximates the values of 0.61, 0.70, and 0.74 $\times$ 10^{-9} recently recorded by Klein (3) for cells of three ascites lymphomas of the mouse and also the value of 0.66 $\times$ 10^{-9} mg. of DNAP reported by Leuchtenberger, Klein, and Klein (4) for nuclei of the ascites lymphoma produced in a DBA strain of mice. The mean value of 0.82 $\times$ 10^{-9} mg/cell obtained by us for the mar-

row of unirradiated human granulocytic leukemia almost duplicates that of 0.84×10^{-9} mg/cell published by Davidson, Leslie, and White (1). The values of cellular DNAP obtained for lymphocytic and granulocytic leukemia correspond with the levels of DNAP per cell for the developing erythroblast in the series designated II and III by us in a study of blood dyscrasias (6). Lower cellular values obtained on bone marrow aspirated from patients receiving x-radiation were similar to the values recorded for series 1 (6), as well as to those recorded for certain patients with pernicious anemia following specific treatment (7).

The figures derived by us on analyses of bone marrow in granulocytic leukemia add evidence to corroborate the claim of Klein and his confreres (3, 4), based on mouse ascites tumors, that DNAP of the "tumor" cell may deviate from that of the normal somatic cell. The fact that tumor tissues from both human and comparative sources give fairly comparable results bespeaks a general biological character for changes in malignancy. The latter has been ascribed to polyploidy (3, 4).

In Chart 1 six lines of regression have been developed in a scatter diagram from the points representing pairs of related DNAP and RNAP recorded in Tables 1 and 2. The first five of these are nearly identical with those found for the splenic lymphocyte of the C57 mouse as is demonstrated in Chart 1. It would appear that three types of blood cell—erythrocyte, lymphocyte, and myelocyte—may have, in certain phases of cellular activity, paired levels of cellular DNAP and RNAP in common. These levels, we believe, have a biological significance not necessarily related to polyploidy.

SUMMARY

1. The average DNAP per cell for samples of bone marrow aspirated from sixteen patients with lymphocytic leukemia was 0.68×10^{-9} mg. Similarly, the average cellular DNAP for fourteen patients with granulocytic leukemia was 0.82×10^{-9} mg/cell. These values were considerably decreased following therapeutic x-radiation.

2. When the paired values of DNAP and RNAP per cell were plotted as points in a scatter diagram on a graph in which DNAP was abscissa and RNAP was ordinate, six lines of regression were developed whose coefficients of correlation were 0.28, 0.36, 0.45, 0.55, 0.69, and 0.85. These values correspond to those similarly derived for the lymphocytes of spleens of the C57BL mouse.

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Enhanced Tumor Transplantability Following Second Transplantation in Mouse of Original Strain

ALBERT E. CASEY AND JOANNE G. CASEY*

(Departments of Pathology of the Birmingham Baptist Hospitals and the Medical College of Alabama, Birmingham, Ala.)

The XYZ factors have been prepared from tumor cells by keeping the tumor tissue frozen for a period of 10 days or longer, either by anaerobic storage (4, 8, 22) or by prolonged desiccation (lyophilization) (4, 24, 25). Ordinarily, tumor cells are killed by this treatment, although the presence of viable tumor cells does not neutralize or diminish the XYZ effect (4, 9, 16, 17). Furthermore, tumor cells have a different thermal death point from the XYZ factors (44° C. for the Z8352 tumor cells and 56° C. for the Z8352 [23] and the Brown-Pearce XYZ factors [13]); and at least two XYZ factors may be obtained from their respective fresh untreated tumors in full potency, by using the supernate from repeated centrifugation for 1 hour at 1200 r.p.m. (14, 22). Despite the presence of the XYZ factors in fresh tumor, a second inoculation of tumor cells 2 weeks after a first does not result in enhanced tumor growth (10). It remains to be proved that the XYZ factors actually influence ordinary tumor growth and spread in the original animal host or even in the inbred strain in which the tumor arose, although it has been proved that the injection of the XYZ factor into the strain of origin 2 weeks before transplant results in enhanced tumor growth and earlier mortality—E 0771 mammary carcinoma in C57BL/6Jax mice (11, 12) and Z8352 tumor in C3H mice (23). To ascertain whether the XYZ factors actually influence tumor growth in the inbred strain of origin E 0771/Jax mammary carcinoma was reinoculated after 12–14 days into a different site in C57BL/6Jax mice. After the second transplant had become large enough for transfer (after 12–17 days), this second transplant was inoculated into BALB/c mice ordinarily resistant to this neoplasm. As controls BALB/c mice were inoculated with tumor taken from C57BL/6Jax controls singly inoculated with E 0771/Jax.

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MATERIALS AND METHODS

Trocar implants of E 0771/Jax into C57BL/6Jax mice resulted in death from tumor growth at 14 days in 50 per cent of the mice. Therefore, emulsions were used when it had been determined that 0.1 cc. of 25 per cent emulsion of E 0771/Jax tumor cells in saline would kill 50 per cent of C57BL/6Jax mice by the 23d day. The mice, C57BL/6Jax and BALB/c, 3–8 months of age when used, and the tumors (E 0771/Jax, 11–14 days old after trocar implant) were obtained in each experiment from the inbred nucleus at the Jackson Memorial Laboratory at Bar Harbor, Maine. Additional mice, C57BR/cdJax × BALB/cJax, hybrids bred in the Baptist Hospital laboratory, were inoculated in parallel experiments not recorded in detail here. The mice were 3–6 months of age and were divided and caged upon receipt into control and experimental groups. There were three experiments (Table 1), and 0.1 cc. of a saline emulsion of tumor cells was injected subcutaneously into the groin in all instances. Tumors were measured twice weekly in three dimensions with calipers.

Experiment I.—E 0771 tumor (from C57BL/6Jax mouse 5405) implanted by trocar in the Jackson Laboratory 12 days before was suspended in normal saline after aseptic removal; 0.1 cc. of a 17 per cent saline cell suspension was injected subcutaneously into the left groin of 20 C57BL/6Jax mice. Twelve days later ten of the mice (those with even numbers) were given subcutaneous injections into the right groin of 0.1 cc. of a 25 per cent saline suspension of E 0771/Jax tumor cells. The tumor (not ulcerated and from C57BL/6Jax mouse 5406) had been injected by trocar at the Jackson Laboratory 24 days before (and from same tumor as 5405). Seventeen days after the second implant of E 0771, a mouse (#5064) with good tumors, neither of which was ulcerated, was killed for transfer. The tumor in the left groin (inoculated 29 days before with 17 per cent emulsion) measured 2.55 × 2.45 × 1.85 cm. and that in the right groin 2.05 × 1.35 × 1.25 cm. The latter tumor (second inoculation, 17 days old) was minced, ground in a mortar, and suspended in normal saline, and 0.1 cc. of a 25 per cent cell suspension was injected subcutaneously into the left groin of four BALB/cJax, three C57BR/cdJax

(resistant to E 0771), and eight C57BR/cd × BALB/c hybrids. Controls for the BALB/c mice were 27 BALB/cJax injected with E 0771 taken from a third C57BL/6Jax mouse implanted by trocar in the Jackson Laboratory.

Experiment II.—C57BL/6Jax mouse (#5835) given inoculations by trocar at the Jackson Laboratory with E 0771/Jax was killed 11 days after transplant and 0.1 cc. of a 30 per cent suspension of tumor cells injected subcutaneously into the right groin of seven C57BL/6Jax mice, nine BALB/cJax mice, and ten cd/c (C57BR/cd × BALB/c hybrids). Fourteen days later one of the mice (C57BL/6Jax mouse #5610) with an unulcerated tumor was killed, and 0.1 cc. of 25 per cent tumor cell suspension in normal saline was injected subcutaneously into the left groin of three C57BL/6Jax mice (even numbers) inoculated right groin subcutaneously with E 0771 (14 days prior). Twenty-seven days after the first and 13 days after the second inoculation one of the doubly inoculated mice (C57BL/6Jax mouse inoculated twice with E 0771/Jax tumor) was killed, and the 13-day-old tumor in the left groin, measuring 1.05 × 0.9 × 0.9 cm. (the tumor in right groin measured 2.55 × 2.2 × 1.55 cm.), was emulsified in normal saline, and 0.1 cc. of a 20 per cent tumor cell suspension injected subcutaneously into the right groin of five C57BL/6Jax mice (5590–5), eight BALB/c mice (5220–5, 5880–1), and into three cd/c mice (5874–6).

Experiment III.—C57BL/6Jax mouse (#5840) inoculated 15 days before by trocar at the Roscoe B. Jackson Memorial Laboratory with E 0771/Jax was killed; the tumor was suspended in normal saline by grinding in a mortar with saline, and 0.1 cc. of a 25 per cent tumor cell suspension was injected subcutaneously in the left groin of ten C57BL/6Jax mice (#5576–85) and into four BALB/cJax mice. Afterwards, a C57BL/6Jax mouse (#5576) inoculated 13 days prior with E 0771/Jax was killed, and the tumor was emulsified in normal saline by grinding in mortar; 0.1 cc. of a 20 per cent suspension of tumor cells was injected subcutaneously into the right groin of five C57BL/6Jax mice previously inoculated with the E 0771/Jax (Nos. 5577, 5579, 5581, 5583, 5585, odd numbers; and into six C57BL/6Jax mice (#5626–31) into five BALB/cJax and into seven cd/c (57BR/cdJax × BALB/cJax hybrids) not previously inoculated. Approximately 27 days after the first and 14 days after the second inoculation, a C57BL/6Jax mouse (# 5583) doubly inoculated with E 0771/Jax tumor was killed (tumor in left groin, 2.55 × 2.0 × 1.55 cm.; and in right, 1.75 × 1.25 × 1.3 cm.); the tumor from the right groin (second inoculation, 14 days old) was emulsified in normal saline, and 0.1 cc. of a 20 per cent suspension of tumor cells injected subcutaneously in the left groin of eight BALB/cJax and six cd/c mice not previously inoculated.

TABLE 1

ENHANCED TUMOR TRANSPLANTABILITY AFTER SECOND TRANSPLANTATION INTO MICE OF ORIGINAL INBRED STRAIN

Growth in BALB/c Mice of E 0771 Mammary Carcinoma Taken from a Second Transplant in C57BL/6Jax Mice

	Transplants into C57BL/6Jax Mice (Original inbred strain)		Transplants into BALB/c mice (Foreign inbred strain)	
Experiment No.	E 0771 once (controls)	E 0771 + E 0771 after 2 weeks	E 0771 from singly inoc. controls	E 0771 taken from 2d inoc. in C57BL/6Jax mice
	Died/Hosts	Died/Hosts	Died/Hosts	Died/Hosts
1	10/10	10/10	1/27	3/4
2	3/3	3/3	1/9	4/8
3	4/4	5/5	0/9	5/8
Subtotal	17/17	18/18	2/45	12/20
			P=0.001 (sig.)	
Other exps.	52/52		2/76	
Total	69/69	18/18	4/121	12/20
			P=0.001 (sig.)	

RESULTS

The results are tabulated (Table 1 and Chart 1). Mice of the C57BL/6Jax strain (in which E 0771 originally arose) had no difference in the cumulative mortality curve in days after inoculation or in the percental accelerant index (22), when inoculated only once with E 0771 or when given a second transplant 12–14 days later.

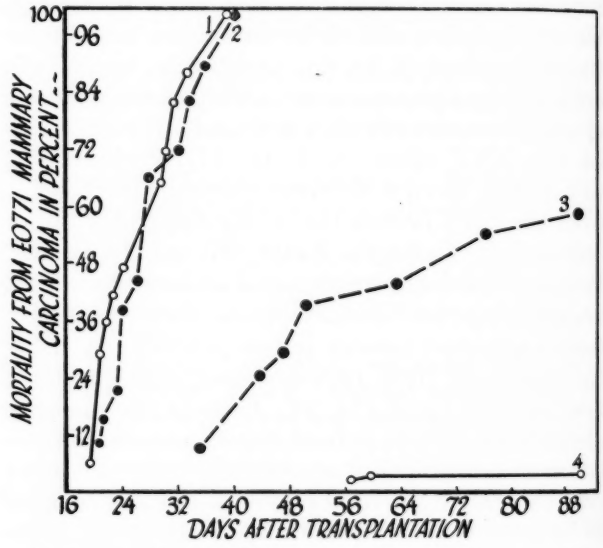

CHART 1.—Enhanced tumor transplantability of a second transplant into mouse of original strain. (1) Cumulative mortality in per cent among seventeen C57BL/6Jax control mice transplanted singly, and (2) among eighteen C57BL/6Jax mice doubly inoculated with E 0771 mammary carcinoma; (3) cumulative mortality among 20 BALB/c mice inoculated with E 0771 carcinoma taken from a second transplant into a C57BL/6Jax mouse; (4) 45 BALB/c controls transplanted with E 0771 carcinoma taken from a first transplant in C57BL/6Jax mice.

In each of the three experiments E 0771 carcinoma taken from a doubly inoculated C57BL/6Jax mouse grew in and killed 50 per cent or more of the resistant BALB/cJax mice without any treatment whatever. On the other hand, E 0771 carcinoma taken for transfer from singly inoculated C57BL/6Jax mice grew but rarely in BALB/cJax mice.

Furthermore, resistant hybrid mice (C57BR/cd × BALB/c) were successfully transplanted in each of the three original experiments with E 0771

from the doubly inoculated C57BL/6Jax mice. The tumor mortality among these hybrids both in the primary and in the serial transfers was about as great as in the pure line BALB/cJax mice. There were fifteen deaths among 57 hybrids thus inoculated in primary and serial transfer, as compared to one death among 22 hybrid controls (transplanted from a singly inoculated C57BL/6Jax mouse; $\chi^2 = 4.6$, $n = 1$, $P = 0.03$, probably sig.). In other experiments this hybrid strain has had the same death rate from E 0771/Jax as the pure BALB/cJax (i.e., 3 per cent).

DISCUSSION

When the growth of tumors is induced in a foreign resistant mouse strain by the use of trypan blue (1), flannel (15), x-ray (18, 26), cortisone (26), or by repeated hemorrhage[1] the tumors do not become adapted to the new hosts and fail to grow in serial transplants. Only the XYZ factors are known to induce permanent adaptations of a tumor to a foreign strain unless the factor described in C3H hybrids is not an XYZ factor (2, 3). This premise is now being tested by serial transfer of the E 0771 tumor in BALB/cJax and cd/c mice. Thus far, seventeen of 54 BALB/c mice have died in serial passages from E 0771 tumor adapted to BALB/c and cd/c mice in the three experiments herein reported ($P = 0.01$—sig.). It would seem, therefore, that an XYZ effect was induced in mice of the original strain by the simple procedure of reinoculating with the same neoplasm in a different site 13–14 days later, even though the doubly inoculated animals did not show any direct evidence of it.

A search of the literature reveals other instances of an XYZ effect when animals bearing spontaneous tumors were given auto-transplants of the same tumor into a different site. Thus, the well known Rous chicken tumor VII, an osteochondrosarcoma (20, 21, 27), and the Brown-Pearce carcinoma of the rabbit (19) were originally transplanted to foreign strains only after doubly transplanting in the original host carrying the spontaneous tumor. It has been reported (10) that the Rous chicken tumor No. 1 was adapted to foreign chickens only after preliminary XYZ treatments. Since inoculations of fowls or rabbits with tumor from the primary site were unsuccessful, the very existence and usefulness of these well known neoplasms were made possible by using for transfer tumor taken from an autograft or by direct XYZ treatment.

[1] A. E. Casey, H. H. Shear, J. G. Casey, F. M. Schabel, Jr., and M. L. Wrenn, unpublished material.

SUMMARY

E 0771/Jax mammary carcinoma was inoculated subcutaneously into the groins of 87 C57BL/6Jax mice (original strain), and each died from tumor growth (100 per cent). There was no difference in the mortality in days after transplant or in the "percental accelerant index" (22), the 50 per cent point in the mortality curve, among the 69 inoculated singly and the eighteen inoculated a second time 12–14 days after the first.

Among BALB/cJax mice given subcutaneous inoculations in the groin of E 0771/Jax mammary carcinoma, four of 121 died (3 per cent) when given tumor taken from C57BL/6Jax mice singly inoculated, whereas twelve of twenty died (60 per cent) when given tumor taken from C57BL/6Jax mice doubly inoculated 12–14 days apart ($P = 0.001$, statistically sig.). The E 0771 tumor from the doubly inoculated mice is being transplanted serially, and thus far in two to four serial passages seventeen of 54 BALB/c mice so inoculated have died from the tumor ($P = 0.01$, sig.).

Among 57 hybrid mice (C57BR/cd × BALB/c) given E 0771 taken from doubly inoculated C57BL/6Jax mice, fifteen have died from the tumor in primary and serial transplants (26 per cent), whereas only one among 22 hybrid controls (4 per cent) inoculated with tumor from singly inoculated C57BL/6Jax mice has died from tumor growth ($P = 0.03$, prob. sig.).

Thus, the resistance of a foreign strain of mice to a tumor from an inbred line has been broken by the single expedient of obtaining the tumor used for transplant from a doubly inoculated mouse.

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Passive Immunization of Mice in Parabiosis against a Transplanted Lymphosarcoma*

NANCY GEISSE FALLS AND ARTHUR KIRSCHBAUM

(Department of Anatomy, College of Medicine, University of Illinois, Chicago, Ill.)

Animals in which a transplanted tumor has regressed usually resist retransplantation of the same tumor; in at least some cases this immunity appears to be tumor-specific (1, 7, 10, 18). Resistance to retransplantation of tumors has been induced in rats and mice by surgical removal of an established graft (1), tourniquet strangulation of the graft (11), injection of nonliving tumor substances prior to grafting (15, 17, 20), or injection before grafting of either normal tissue or red blood cells foreign to the host (2, 12, 13). Whether circulating antibodies are associated with such resistance (immunity?) and whether antibodies may passively immunize a susceptible host are problems which have been analyzed to a degree as indicated below.

Serum from mice or rabbits actively immunized by injecting mouse leukemic cells inactivated the living antigenic cells *in vitro* so that their ability to transmit the disease was lost (8, 21). Immunity against leukemic cells arising within the strain has been produced in inbred mice by administering sublethal doses of cells prior to inoculation with massive doses. This immunity was passively transferred to susceptible hosts by implantation of spleen or liver from immune donors (12).

Parabiotic studies have on the one hand indicated transfer of immunity to normally susceptible animals (4, 9), whereas other reports appear to demonstrate the contrary (6, 14), to the extent that the immune condition was actually reported to have been converted to susceptibility (5). Bichel and Holm-Jensen have shown that such seemingly conflicting reports might stem from the genetic type of animals used. By means of labeling red blood corpuscles it was found that the crossed blood flow in homogenetic pairs was approximately 100 times that in pairs that were heterogenetic (3). Immunity was not transferred if the parabionts were heterogenetic, whereas homogenetic parabionts were passively immunized (4). It was concluded that large quantities of antibodies must be transferred to obtain passive immunity and that attempts at passive immunization by injection of serum and blood and by heterogenetic parabiosis have been unsuccessful because of insufficient antibody exchange.

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MATERIALS AND METHODS

Induced regression of Gardner lymphosarcoma, immunity to retransplanted tumor tissue.—It was the purpose of the present study to determine whether a transplanted tumor which grows progressively would evoke immunity to retransplantation by its induced regression and whether such immunity could be transmitted to members of the same strain in parabiosis. In Bichel and Holm-Jensen's experiments (4) tumor immunity was passively transferred from animals in which spontaneous regression of the transplants had occurred.

When the Gardner lymphosarcoma (19) of strain C3H origin (also known as 6C3HED) is transplanted subcutaneously by trocar into CBA mice (obtained from Dr. L. C. Strong and inbred in this laboratory since 1941), a palpable tumor appears at the inoculation site within 8 days. This tumor grows progressively to kill the host within 30 days. The 26th–40th transfer generations carried in this laboratory were used in these experiments. The tumor was obtained originally from Dr. George Moore, then at the University of Minnesota, in February, 1951. In CBA mice 95 per cent successful takes are obtained. The tumor is radiosensitive. At 10 days following transfer, when the tumor is 1 cm. in diameter, 600 r of x-rays directed to the tumor (remainder of the animal lead-shielded) will induce regression within 4–9 days in 50 per cent of the cases.

Thirty-two mice in which tumor regression had been induced by x-radiation were subjected to a challenging reinoculation of the lymphosarcoma. Such transplants were made at various periods of time from 10–35 days following complete regression. Results are shown in Table 1.

Once the tumor had regressed it did not reappear, and, of the 32 mice tested, 29 were immune to retransplantation of the same tumor. This immunity was demonstrable up to 105 days after tumor regression. Of twelve previously untreated CBA mice (Table 1) inoculated with the same tumor tissue, all succumbed to the disease within 12–48 days (average, 23 days). Although CBA mice are genetically distinct from the C3H strain of tumor origin, immunity to the Gardner lymphosarcoma may also develop in C3H mice. Stoerk and Emerson (19) reported that in riboflavin-deficient C3H mice the Gardner lymphosarcoma regressed, did not reappear, and the animals were subsequently resistant to retransplantation for at least 60 days.

TABLE 1

GROWTH OF GARDNER LYMPHOSARCOMA IN MICE IN WHICH A PREVIOUS TRANSPLANT HAD REGRESSED FOLLOWING IRRADIATION AND IN UNTREATED MICE

Regression was induced by 600 r of x-rays. Both groups of mice were of the same age and were inoculated with the same tumors.

Group	No. mice inoculated	Time after regression before challenging transplant (days)	No. immune mice
Mice in which tumor had regressed	6	10–15	6
	6	18	6*
	17	20–30	14
	3	35	3
	32	10–35	29
Previously untreated controls	12		0

* 105 days later these mice were still immune.

Transfer of immunity by parabiosis.—In the present experiments immune CBA animals were placed in parabiosis with untreated CBA mice 15–45 days after a challenging inoculum into the immune mice. Litter-mates were not used, although CBA mice of the same weight, age, and sex were joined in parabiosis.

The method of parabiosis was that described by Sauerbruch and Heyde (16); it was an open coelomic method in which the peritoneum and abdominal muscles were united with one continuous suture. Another continuous suture united the skin of the two from the scapula to the crest of the ilium. Small strips of adhesive tape were used to bind together the inner forelegs, and similar tapes were applied to the tails to prevent strain on the sutures. Aseptic technic was employed. Skin healing was well established by the 5th postoperative day. At this time both parabionts were given subcutaneous inoculations of the Gardner lymphosarcoma. Only those animals which survived in parabiosis 15 or more days after transplantation are considered for analysis of data. There were fourteen such pairs whose survival ranged from 15 to 123 days after tumor transfer, the average being 34 days. Controls were twelve sets of non-immune CBA parabionts which were inoculated with the same donor tumors as the test parabionts. These pairs averaged 30 days in parabiosis after transplantation, with a range of 20–36 days (Table 2).

Of the fourteen cases where actively immunized mice were in parabiosis with an untreated partner, in nine instances the latter resisted transplantation. In three sets only the untreated partner developed a tumor after inoculation, and in two pairs tumor growth occurred in both parabionts (Table 1). In eleven of the twelve control pairs, where both parabionts were untreated susceptible CBA mice, tumors developed in both members, and in one pair one of the animals did not develop a tumor (Table 2).

Immune mice in parabiosis with susceptible animals showed no local tumor upon reinoculation, whereas the susceptible member of the pair developed a barely palpable growth which subsequently regressed (nine of fourteen cases). It appears that the susceptible mice passively acquired sufficient resistance from the immune parabiont (by transfer of antibodies?) to check the growth of the tumor until their own defense mechanism destroyed it.

TABLE 2

PASSIVE TRANSFER OF IMMUNITY BY PARABIOSIS

Previously Untreated CBA Mice Were Joined to CBA Mice in Which a Gardner Lymphosarcoma Transplant Had Been Caused To Regress or, as Controls, to Untreated CBA Mice

TEST

IMMUNE IN PARABIOSIS WITH SUSCEPTIBLE MOUSE*

Total no. parabiotic pairs	No. in which previously susceptible parabiont resisted transplantation
14	9

No growth in immune, no growth in susceptible parabiont: 9
No " " " , growth " " " " : 3
Growth in both immune and susceptible : 2

CONTROL

Susceptible in parabiosis with susceptible mouse†

12	1

No growth in either: 11
Growth in only one: 1

* Survival time of 15–123 days—Average, 34 days.
† Survival time of 20–36 days—Average, 30 days.

That the effect of x-rays in promoting tumor destruction was not the stimulus to immune reaction was demonstrated in the following manner. Twenty-one mice were simultaneously bilaterally

inoculated with the Gardner lymphosarcoma. All 21 had large bilateral, subcutaneous tumors of similar size at death. A series of twenty mice were inoculated with the tumor on only the left side 10 days before a second inoculation was made on the right side. At autopsy only nine of the twenty had bilateral growths; the remaining eleven had enormous growths on the left but no tumor on the right side.

Apparently the Gardner lymphosarcoma provokes the development of resistance during its progressive growth in CBA mice, but the degree of resistance is ordinarily not sufficient to inhibit growth of the implant, whose presence caused the immune reaction. A second tumor inoculum is met with the already developed resistance, which, in 55 per cent of the cases, prevented progressive growth of the second transplant. X-radiation may merely cause the initial tumor to regress, allowing the animal to survive with its previously developed immunity. However, 90 per cent of the mice whose tumors had been caused to regress by x-radiation resisted retransplantation.

SUMMARY

A lymphosarcoma of strain C3H origin (Gardner lymphosarcoma, also known as 6C3HED) grew progressively when transplanted into CBA mice. When tumor regression was induced by x-radiation the mice were resistant to retransplantation of the same tumor. This resistance could be demonstrated up to 105 days after tumor regression and could be transmitted to previously susceptible animals of the same strain by parabiosis. Radiation effects per se may not have been responsible for producing the immune response, since 55 per cent of nonirradiated animals with rapidly growing tumors resisted a second tumor graft.

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Metastatic Frequency of Spontaneous Mammary Carcinoma in Mice Following Biopsy and Following Local Roentgen Irradiation

SIGVARD KAAE

(The Radium Centre, Copenhagen, Denmark)

The risk of spread of malignant tumors following biopsy has been the subject of numerous investigations (2), while only a few workers have been concerned with this risk following local roentgen irradiation. Yamamoto (4) found an increased frequency of metastasis to bone following local roentgen irradiation of the Kato rabbit sarcoma with doses of 600–1,800 r. Kaplan and Murphy (3) investigated the frequency of remote metastases following local roentgen irradiation of the Bagg-Jacksen mammary carcinoma 755 transplanted into C57BL mice. Pulmonary metastases occurred in 44 per cent of 62 mice irradiated locally, as against 9.6 per cent of 52 controls.

TABLE 1

FREQUENCY OF METASTASES AND OF MULTIPLE MAMMARY CARCINOMAS (>1 TUMOR) IN ALL MICE

		Mice with metastases		Mice with multiple tumors	
	No.	No.	Per cent	No.	Per cent
Biopsy mice	132	15	11.4	30	22.7
Irradiated mice (1,000 r)	53	9	17.0	10	18.9
Irradiated mice (2,000 r)	82	21	25.6	30	36.6
Irradiated mice (total)	135	30	22.2	40	29.6
Control mice	92	5	5.4	34	37.0

Later, Essen and Kaplan (1) showed that the effect of local irradiation was due to a transient local disturbance of the host-tumor relationship, probably concerned with facilitating entry of the tumor cells into blood vessels.

MATERIALS AND METHODS

The author studied the incidence of metastases following biopsy and following local roentgen irradiation of spontaneous mammary carcinomas in DBA mice, inbred through more than 40 generations. The incidence of spontaneous mammary carcinoma is very high in females of this strain.

The material was divided into three groups. The mice in Group 1 were examined by biopsy with a rotating trephine under local anesthesia, usually when the tumor had reached the size of 8–10 mm. Since the biopsy specimens showed carcinoma in 96 per cent of the cases, in these cases at least the trephine had been in the tumor. The mice in Group 2 received local roentgen irradiation, usually when the maximum diameter of the tumor was 8–10 mm. During the irradiation of the tumor the remainder of the body was carefully shielded with lead, 4 mm. thick. The irradiation conditions were as follows: 180 kv., 3 mm. Al filter, HVL = 0.3 mm. Cu, and 40 cm. focal-skin distance. The dose was in some cases 1,000 r and in others 2,000 r (r measured in air). Group 3 comprised control mice. All the mice lived under carefully controlled identical conditions, and the tumors were measured each week. All mice were carefully autopsied, including measurement, weighing, and microscopic study of the tumor. The microscopic studies showed carcinoma of the mammary type in all cases. Moreover, the mice were carefully examined for metastases and multiple mammary tumors. Remote metastases occurred only in the lungs and, in a few cases, in the mediastinum.

The mice in the three groups were not selected in any way. First the biopsy experiments were carried out and then the irradiation experiments. The control experiments were performed simultaneously. The experiments were continued for 4 years and 8 months. To ascertain that no alteration—especially with regard to spontaneous metastasis—had taken place during this period, the metastatic frequency for the first half and for the latter half of the controls was analyzed. This revealed three metastases in 46 mice during the first half and two in 46 during the latter half. Since no demonstrable change had taken place in the frequency of spontaneous metastasis, all the controls are comparable to the biopsy group as well as to the irradiated group.

RESULTS AND DISCUSSION

A survey of the mice in all three groups is presented in Table 1. Regional lymph node metastases were not found in any case. Many mice, however, developed several, apparently primary, mammary tumors. As it cannot be ruled out that some of them were, in fact, regional lymph node metastases, the incidence of multiple mammary carcinomas at death was analyzed. The incidence of multiple tumors was not higher in the groups which had biopsy or local irradiation than in the control group. This finding militates against a higher incidence of regional lymph node metastases following biopsy or roentgen irradiation. However, minor differences may have escaped detection in an analysis of this nature.

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Remote metastases were observed in the lungs and, in three cases, in the mediastinum without simultaneous metastatic deposits in the lungs. Other organs were not affected.

It will be seen from Table 1 that metastases were more common in the biopsy than in the control group (11.4 per cent, compared to 5.4 per cent), but the difference is not significant, being only 1.6 times the standard deviation. In the irradiated group, on the other hand, metastases were considerably more common than in the control group (22.2 per cent, compared to 5.4 per cent). This difference is significant—3.8 times the standard deviation. Metastases appear to be more common following a dose of 2,000 r than one of 1,000 r. Moreover, in the irradiated group the pulmonary metastases were usually numerous and large, whereas in the control group they were, on the whole, few and small.

TABLE 2

FREQUENCY OF METASTASES AND OF MULTIPLE MAMMARY CARCINOMAS (>1 TUMOR) IN SELECTED MICE

Only mice with tumors with a maximum diameter of 8–10 mm. at the institution of the experiment were considered

		Mice with metastases		Mice twih multiple tumors	
	No.	No.	Per cent	No.	Per cent
Biopsy mice	81	7	8.6	22	27.2
Irradiated mice (1,000 r)	30	4	13.3	4	13.3
Irradiated mice (2,000 r)	51	14	27.5	15	29.4
Irradiated mice (total)	81	18	22.2	19	23.5
Control mice	66	3	4.5	23	34.8

Considering the difficulty of obtaining uniform groups for comparison in the case of spontaneous tumors, as contrasted with transplanted growths, a further analysis proved necessary. To this end, those cases in which the tumor had a maximum diameter of 8–10 mm. at the institution of the experiment were selected for a comparison of the survival times. Table 2 gives the frequency of metastasis and of multiple tumors for these more uniform groups. This showed the same ratios between groups as Table 1. Metastases were found to be somewhat more common in the biopsy group (8.6 per cent as against 4.5 per cent in the control group) and, quite particularly, in the locally irradiated group (22.2 per cent as against 4.5 per cent in the control group).

The mice ranged in age from 5 to 25 months at the beginning of the experiments, and the age distribution was not uniform in the three groups. Mice of different age groups may show a different tendency to metastasis, and, moreover, the shorter survival of the older mice must be assumed to give fewer metastases. Therefore, the groups were divided into more uniform age ranges to study the incidence of metastases. Table 3 shows the findings for mice aged 11–15 months. The data conform exactly with the findings shown in Tables 1 and 2; therefore, the difference in the metastatic frequency cannot be due to the slight differences in age distribution among the three groups.

TABLE 3

METASTATIC FREQUENCY IN MICE AGED 11–15 MONTHS AT THE INSTITUTION OF THE EXPERIMENT

		Mice with metastases	
	No.	No.	Per cent
Biopsy mice	36	5	13.9
Irradiated mice	43	11	25.6
Control mice	34	2	5.9

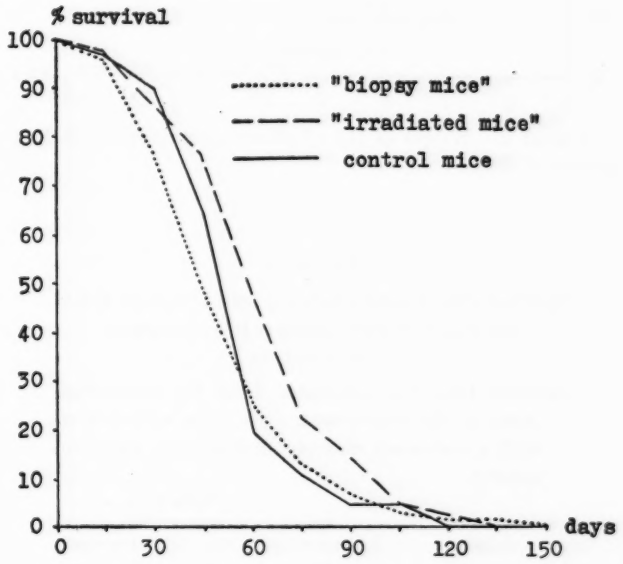

CHART 1.—Survival time of the mice in all three groups calculated from the time when the maximum diameter of the tumors was 8–10 mm.

A difference in survival time in the three groups might influence the metastatic frequency. Particularly, a longer survival in the locally irradiated group might be expected to allow a higher metastatic frequency. The survival time is plotted on Chart 1 which shows that the survival of the mice in the biopsy group is, if anything, somewhat shorter than that in the control group. In the irradiated group, the survival time is unmistakably prolonged. This conforms with the arrest and even regression of tumor growth occurring after roent-

gen irradiation; tumor growth does not occur again for 2 or more weeks. Despite the longer average survival in the irradiated group, the tumors weighed somewhat less than the tumors in the control group (Chart 2). The weights, however, must be interpreted with caution, since many tumors ulcerate, showing infection and sometimes marked necrosis and decrease in weight. The frequency of ulceration, however, is approximately the same in all three groups—64 per cent among 81 mice of the biopsy group, 65 per cent among 81

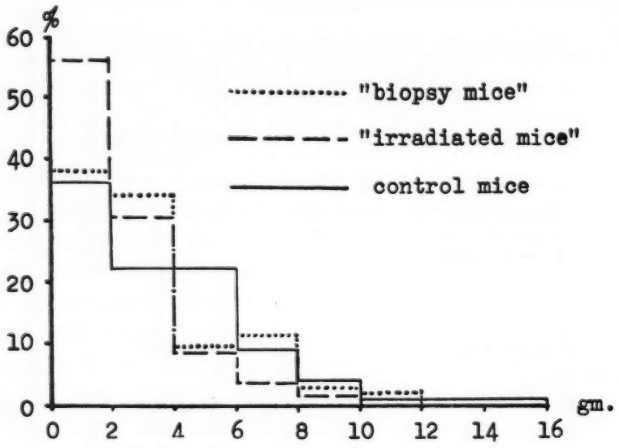

CHART 2.—Weight of the spontaneous tumors in all three groups at death.

TABLE 4

METASTATIC FREQUENCY IN THE LOCALLY IRRADIATED GROUP CLASSIFIED ACCORDING TO SURVIVAL

Survival time was calculated from the commencement of the experiment; only mice with tumors with a maximum diameter of 8–10 mm. were considered.

Survival time (days)	No. mice	Mice with metastases No.	Per cent	
Less than 46	20	2	10	
46–60	22	4	18.2	
61–75	21	7	33.3	30.8
Greater than 75	18	5	27.8	
Total	81	18	22.2	

mice of the irradiated group, and 61 per cent among 66 mice of the control group.

To ascertain whether the increased metastatic frequency in the irradiated group was due exclusively to the prolonged survival time, the frequency of metastasis at different survival times in this group was analyzed in more detail. In addition, the metastatic frequency was computed assuming the irradiated mice had survived for only the same length of time as the controls. If the increased metastatic frequency in the irradiated group had been due exclusively to a longer survival time, this computation would have given the same metastatic frequency as in the control group. Table 4 gives a rough classification of the mice in the irradiated group according to survival, illustrating also the metastatic frequency. Table 5, similarly, gives the distribution of the survival times for the controls as well as the computed frequency of metastasis in the irradiated mice, assuming the latter had survived for the same length of time. This calculation gave a corrected metastatic frequency of 17.7 per cent in the irradiated group, compared to 4.5 per cent in the control group. A further classification into ten survival times with only 10 days in each group also gives a corrected

TABLE 5

CLASSIFICATION OF CONTROL MICE ACCORDING TO SURVIVAL TIME, STATING THE METASTATIC FREQUENCY

The table also shows the metastatic frequency in the irradiated group at different survival times and the computed number of metastases, if the survival time of the irradiated mice had been of the same distribution as in the control group.

Survival time	No. control mice	Mice with metastases No.	Per cent	Metastatic frequency in irradiated group	Computed expected number
Less than 46 days	24	2		10	2.4
46–60 days	29	1		18.2	5.3
More than 60 days	13	0		30.8	4.0
Total	66	3	4.5		11.7:17.7%

metastatic frequency of about 17 per cent in the irradiated group. The difference between 17 per cent and 4.5 per cent is significant. In other words, the increased metastatic frequency in the locally irradiated group is due only to a small extent to the prolonged survival time, but mainly to a real increase in the metastatic frequency following local roentgen irradiation of the tumor.

Kaplan and Murphy, studying a transplanted mouse tumor, found the metastatic frequency to be increased following local roentgen irradiation. The present experiments have confirmed that this also applies to spontaneous mouse carcinomas. This means that if roentgen irradiation of cancer fails to produce complete disappearance of the tumor, metastasis may occur earlier than if no irradiation had been administered. Of course, mouse experiments do not permit conclusions regarding human cancer, but this possibility must be borne in mind. In this respect there may be

marked differences between cancers of different varieties and sites.

The present experiments did not reveal a significantly increased metastatic frequency following biopsy. At any rate, the tendency to metastasis following biopsy was not so marked as that following local roentgen irradiation.

SUMMARY

Local roentgen irradiation of spontaneous mammary carcinomas in DBA mice with doses of 1,000–2,000 r considerably increased the incidence of pulmonary metastases, as compared to a control group (22.2 per cent against 5.4 per cent). This increased incidence of pulmonary metastases was due only to a small extent to a longer survival in this group, but primarily to a real increase in the metastatic tendency caused by the irradiation. Removal of biopsy specimens did not significantly increase the incidence of metastases (11.4 per cent, compared to 5.4 per cent in the control group).

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The Production of L(+) Lactic Acid by Ehrlich Mouse Ascites Tumor Cells*

MYRON BRIN

(Cancer Research Institute of the New England Deaconess Hospital, Boston, Mass.)

The Ehrlich mouse ascites tumor has been the subject of considerable biochemical interest during the last few years. Klein (11) has studied the RNA and DNA metabolism of these cells, and Christensen and Riggs (7) have investigated the ability of this free-growing tumor cell to set up concentration gradients of amino acids across the cell membrane. This laboratory has recently become interested in the metabolism of the ascites tumor as well as the physiology of the tumor-host relationship (Brin *et al.* [5], McKee *et al.* [13–15]), and has shown that the ascites tumor has a very high rate of glycolysis (Brin [3]). It was considered of interest, therefore, to isolate and characterize the lactic acid obtained through glycolytic conversion from glucose by the ascites tumor cells.

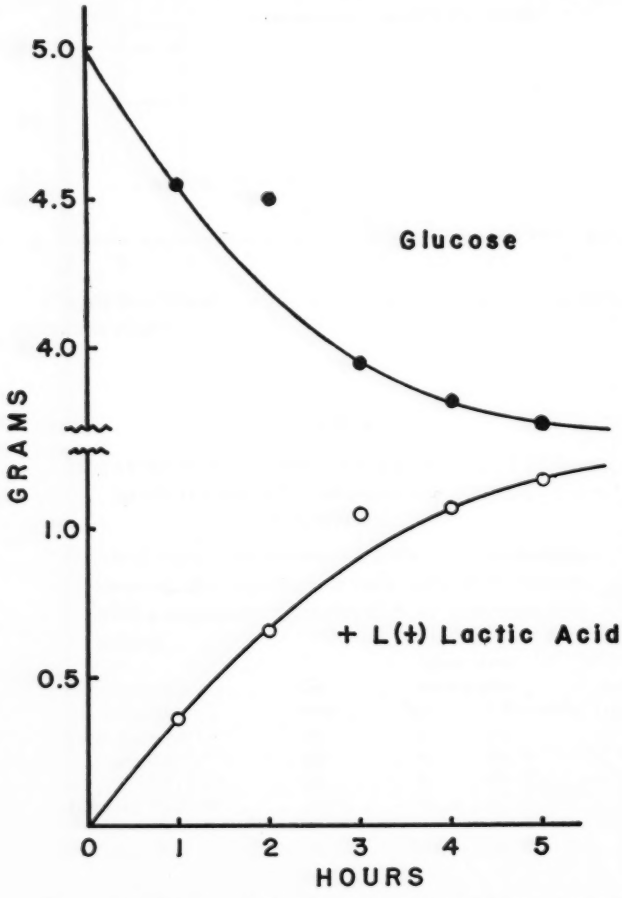

CHART 1.—The production of L(+) lactic acid from glucose by the Ehrlich mouse ascites tumor cells.

MATERIALS AND METHODS

Seventeen C3H brown mice were inoculated with 0.2 ml. ascites suspension, and the tumor was permitted to develop for 7 days. Upon sacrificing the animals, two animals were discarded due to gross hemorrhagic ascites. The yield of fluid and cells from the remaining fifteen mice was 100 ml. The cells were spun down and resuspended in 1,000 ml. of Krebs phosphate saline, pH 7.4 (NaCl, 0.115 M; KCl, 0.004 M; $MgCl_2$, 0.005 M; $Na_2HPO_4 - NaH_2PO_4$, 0.020 M) containing 5 gm. glucose. The final hematocrit was 1.8. The suspension was poured into a 2-liter round-bottom flask which was then placed in a 37° C. bath, stoppered with nonabsorbent cotton, and maintained under nitrogen for 5 hours. The flask was stirred every 15 minutes, and aliquots were taken hourly for the determination of lactate by the method of Barker and Summerson (1). The lactic acid was removed from the system as described by Brin (4), and the zinc lactate thereby recovered was then recrystallized without the use of alcohol (Brin *et al.* [6]). The zinc lactate was characterized by water of crystallization, optical rotation, and carbon, hydrogen, and ash analyses.

RESULTS

The rate of glycolysis by the tumor is shown in Chart 1. Evidently the tumor metabolized at a constant rate for 3 hours and then leveled off with the production of 1.17 gm. of lactic acid at 5 hours. This is equivalent to 1.81 gm. of hydrated zinc lactate. Of this, 1.26 gm. of zinc salt was isolated, or about 70 per cent of the total.

A comprehensive survey of the literature revealed that Warburg (16) had previously characterized the lactic acid produced by Jensen rat

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sarcoma slices. Since this was the only other study uncovered which identified the lactate isomer produced by a tumor, the data are included for comparison. The combined data are presented in Table 1. It should be noted that the rotation of the zinc salt is opposite that of the free acid. Chart 2 demonstrates that the absolute rotation of zinc lactate varies markedly with the concentration of anhydrous salt in solution (17). It is observed that the absolute rotation for each tumor lies directly on the theoretical curve.

The lactate produced by the Ehrlich mouse ascites tumor is evidently L(+) lactate, the same isomer as that produced by the Jensen rat sarcoma and identical with that produced by muscle.

TABLE 1

CHARACTERISTICS OF ZINC LACTATE PRODUCED BY ANIMAL TUMORS

Tumor	Water of crystallization (per cent)	Specific* rotation degrees	Elemental analysis* Carbon	Hydrogen (per cent)	Ash	Isomer
Ehrlich mouse ascites	12.9	− 8.0†	29.6	4.2	34.4	L(+)
Jensen rat (16)	12.6	−10.2‡	29.6	3.7	33.5	L(+)
Theoretical (for L(+) isomer)	12.9	− 8.0†	29.6	4.1	33.4	L(+)

* Anhydrous salt.
† Salt concentration, 2.5 per cent.
‡ Salt concentration, 1.55 per cent.

DISCUSSION

The production of lactic acid by animal organisms as well as the evaluation of molecular isomerism has been of interest to biochemists for the last century. Although the discovery of lactic acid has been credited to Scheele in 1780 (18) and often to Berzelius in 1806 (9), it was not until 1848 that Engelhardt (10) differentiated between the racemic lactate of sour milk and the sarcolactate of muscle by the differing solubility, water of crystallization, and decomposition points of the calcium and zinc salts. The concept of isomerism in lactate was introduced finally in 1873 by Wislicenus (19) after he evaluated the optical rotation of the various salts. Succeeding studies have demonstrated that L(+) lactic acid is the form found in normal animal tissues (2, 12), and the present study confirms the previous study by Warburg that it is L(+) lactate that is produced in glycolyzing tumor tissue. These data lend further support to concurrent studies in our laboratory to the effect that the Ehrlich mouse ascites tumor metabolizes L(+) lactic acid far more rapidly than D(−) lactic acid (5). The production of natural lactic acid by tumors is of further interest in the light of recent work by Wiltshire (18) demonstrating the presence of only the natural form of glutamic acid in these tissues.

SUMMARY

Ehrlich mouse ascites tumor cells were incubated with glucose under an atmosphere of nitrogen for 5 hours, after which time the lactic acid formed was extracted, crystallized, and characterized as the zinc salt. Data on water of crystallization, carbon, hydrogen, and ash analyses, and optical rotation indicate that the tumor produced L(+) lactate.

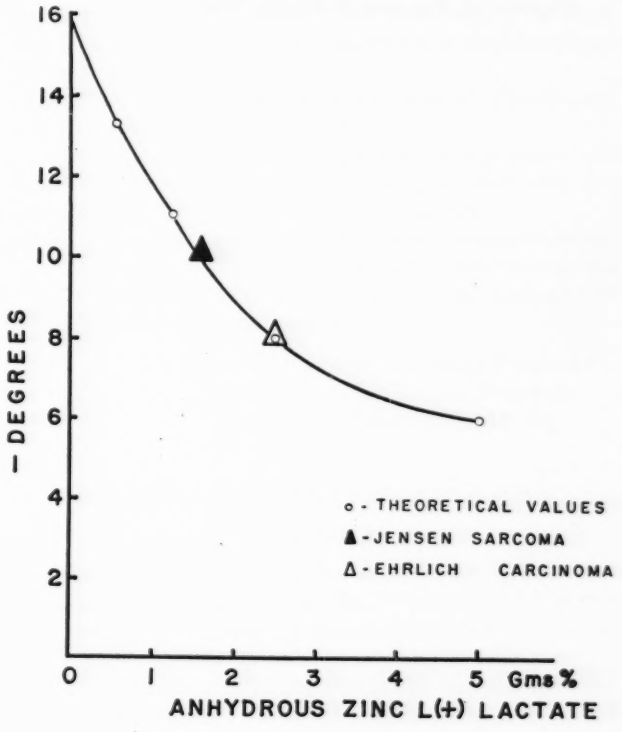

CHART 2.—The relation between the concentration of anhydrous zinc lactate and the specific rotation of the solution (17).

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Announcements

The American Association for Cancer Research, Inc., Annual Meeting

The 45th annual meeting of the American Association for Cancer Research, Inc., will be held in Haddon Hall, Atlantic City, N.J., on April 10–12, 1954.

Abstracts of papers accepted for presentation at the meeting will be published and distributed to members of the Association before the meeting as *Proceedings of the American Association for Cancer Research*. Copies of the *Proceedings* also will be available for sale at the time of the meeting.

The following regulations concerning abstracts and presentation of papers have been established by the Program Committee and approved by the Board of Directors of the Association. Failure to comply with the requirements will result in the rejection of abstracts. Papers represented by rejected abstracts will not be considered by the Program Committee, and such abstracts will not be published in the *Proceedings*. No abstract of a paper accepted by the Program Committee, either for oral presentation at the meeting or for presentation by title, can be withdrawn from publication or altered in composition by the author.

a) Four copies of the abstract are to be sent to the Chairman of the Program Committee, Dr. G. B. Mider, National Cancer Institute, Bethesda 14, Maryland.

b) The abstracts are required by January 4, 1954, and must be postmarked no later than December 30, 1953.

c) A member of the Association may submit only one paper of which he is the principal author and one paper of which he is the co-author. This rule also applies to a nonmember. Abstracts from nonmembers must be introduced by a member; no more than two such introductions may be made by one member. Authors may request that their paper be "read by title." Such requests will be recorded on the printed program.

d) Abstracts must adequately describe the objectives and results of the research so that the Committee may be able to judge the quality, originality, and completeness of the work. Authors must accept sole responsibility for their statements and should exercise great care in the preparation of the abstracts; galley proof cannot be sent because of time limitations.

e) A request for presentation of a paper at the meeting of the American Association for Cancer Research, Inc., implies that the author has not presented, or is not planning to present, the same subject matter at a meeting of another national scientific organization.

f) Abstracts must not be longer than 250 words. The style used in previous abstracts (see *Proceedings*, April, 1953) is to be followed. The title of the paper is to be followed by the names of the authors, their university, laboratory, or hospital, and its location. The names of nonmembers should be marked with an asterisk; when needed, the name of the member introducing the paper is to be included, i.e. "(Introduced by A. J. Dee)." Abstracts must be typewritten, double-spaced on $8\frac{1}{2} \times 11$-inch paper.

g) If reprints of abstracts are desired, the request must be sent with the abstract.

The time allowed for presentation of any paper will be 10 minutes. Projectors will be provided only for lantern slides $3\frac{1}{4} \times 4$ inches or 2×2 inches.

By action taken at the 1952 Annual Business Meeting of Members of the Association, a registration fee of $3.00 will be charged each member or nonmember attending the scientific sessions.

Hugh J. Creech, *Secretary-Treasurer*
The Institute for Cancer Research
Fox Chase, Philadelphia 11, Pa.

Grants for Scholars in Cancer Research

Applications for grants for Scholars in Cancer Research are being accepted by the Committee on Growth of the National Research Council, acting for the American Cancer Society.

These awards are designed to bridge the gap between the completion of fellowship training and the period when the young scientist has thoroughly demonstrated his competence as an independent investigator. A grant of $18,000, payable over 3 years, will be made to each Scholar's institution as a contribution toward his support, his research, or both. Each institution may submit more than one application. These grants are not restricted to the support of individuals who have held American Cancer Society Fellowships. Applications should be submitted by institutions on behalf of a candidate prior to January 1, 1954.

Additional information may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

Book Reviews

Blood Cells and Plasma Proteins: Their State in Nature. JAMES L. TULLIS (Ed.). New York: Academic Press, Inc., 1953. Pp. 436. $8.50.

The second in a projected series of memoirs from Harvard's University Laboratory of Physical Chemistry Related to Medicine and Public Health, this volume is a compilation of material given at a group of seminars at Harvard in the spring of 1951. The subject matter covers seven main categories: a general discussion of plasma proteins, the components involved in blood coagulation, the factors concerned with immunity, erythrocytes, leukocytes, plasma enzymes, and blood lipoproteins. The 28 chapters were written by 29 authors, among whom are many of the leading investigators in these fields.

Essentially, the chapters are short reviews of the current thinking in each of the various areas. Included in some of the chapters is a fair amount of recent experimental data. Some sections are rather broad and sketchy in nature, as, for example, "The Origin, Properties, Function, and Preservation of White Bood Cells," a 24-page chapter by James L. Tullis. Others are more limited in scope as, for example, the chapter on "Linkages between Proteins and Lipids" by Jordi Folch-Pi which covers only 3½ pages. Considerable space is given to discussion of historical background. An especially entertaining chapter by Professor E. J. Cohn (progenitor of much of the work in this volume) reviews the development of knowledge about blood from its beginnings in 16th century Padua.

Inevitably, in a book of this sort, there is unevenness in the style, purpose, and profundity of the various authors. And, because these areas of science are now so urgently under investigation, it is all but impossible to present something between hard covers that is truly up-to-date. Nevertheless, this book does fill a need and it should be most welcome, both to active investigators and to those seeking only a general orientation in a rapidly expanding province. The bibliographies, though lacking titles, are helpful, the index is adequate, and the over-all tone of the book is engagingly informal.

WILLIAM S. BECK

Atomic Energy Project
School of Medicine
University of California at Los Angeles